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<p>(21) International Application Number: PCT/US94/02536 (22) International Filing Date: 9 March 1994 (09.03.94) (30) Priority Data: 08/034,949 22 March 1993 (22.03.93) US (71) Applicant (for all designated States except US): THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF THE ARMY [US/US]; c/o Intellectual Property Counsel of the Army Office of The Judge Advocate General, DA, Suite 400, 901 North Stuart Street, Arlington, VA 22203-1837 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): REID, Robert, H. [US/US]; 10807 McComas Court, Kensington, MD 20895 (US). BOEDEKER, Edgar, C. [US/US]; 7505 Bybrook Lane, Chevy Chase, MD 20815 (US). (74) Agent: BELLAMY, Werten, F., W.; Intellectual Property Law Division, Office of The Judge Advocate General, Suite 400, 901 North Stuart Street, Arlington, VA 22203-1837 (US).</p>		<p>(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.</p>
<p>(54) Title: VACCINES AGAINST DISEASES CAUSED BY ENTEROPATHOGENIC ORGANISMS USING ANTIGENS ENCAPSULATED WITHIN BIODEGRADABLE-BIOCOMPATIBLE MICROSPHERES (57) Abstract This invention is directed to oral parenteral and intestinal vaccines and their use against diseases caused by enteropathogenic organisms using antigens encapsulated within biodegradable-biocompatible microspheres.</p>		

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1 VACCINES AGAINST DISEASES CAUSED BY ENTEROPATHOGENIC
2 ORGANISMS USING ANTIGENS ENCAPSULATED WITHIN
3 BIODEGRADABLE-BIOCOMPATIBLE MICROSPHERES

4 I. GOVERNMENT INTEREST

5 The invention described herein may be
6 manufactured, licensed and used by or for governmental
7 purposes without the payment of any royalties to us
8 thereon.

9 II. CROSS REFERENCE

10 This application is a continuation-in-part of
11 U.S. Patent Application Serial No. 07/867,301 filed
12 April 10, 1992 which in turn is a continuation in part
13 of U.S. Patent Application Serial No. 07/805,721 which
14 in turn is a continuation-in-part of U.S. Patent
15 Application Serial No. 07/690,485 filed April 27, 1991,
16 which in turn is a continuation-in-part of U.S. Patent
17 Application Serial No. 07/521,945 filed May 11, 1990,
18 which in turn is a continuation-in-part of U.S. Patent
19 Application Serial No. 07/493,597 filed March 15, 1990,
20 which in turn is a continuation-in-part of U.S. Patent
21 Application Serial No. 06/590,308, filed March 16,
22 1984.

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1 III. FIELD OF THE INVENTION

2 This invention relates to parenteral and
3 oral-intestinal vaccines against diseases caused by
4 enteropathogenic organisms using antigens encapsulated
5 within biodegradable-biocompatible microspheres
6 (matrix).

7 PHASE I

8 IV. BACKGROUND OF THE INVENTION

9 Most infectious agents have their first
10 contact with the host at a mucosal surface; therefore,
11 mucosal protective immune mechanisms are of primary
12 importance in preventing these agents from colonizing
13 or penetrating the mucosal surface. Numerous studies
14 have demonstrated that a protective mucosal immune
15 response can best be initiated by introduction of the
16 antigen at the mucosal surface, and parenteral
17 immunization is not an effective method to induce
18 mucosal immunity. Antigen taken up by the
19 gut-associated lymphoid tissue (GALT), primarily by the
20 Peyer's patches in mice, stimulates T helper cell (T_H)
21 to assist in IgA B cell responses or stimulates T
22 suppressor cells (T_s) to mediate the unresponsiveness
23 of oral tolerance. Particulate antigen appears to
24 shift the response towards the (T_H) whereas soluble
25 antigens favor a response by the (T_s). Although

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1 studies have demonstrated that oral immunization does
2 induce an intestinal mucosal immune response, large
3 doses of antigen are usually required to achieve
4 sufficient local concentrations in the Peyer's
5 patches. Unprotected protein antigens may be degraded
6 or may complex with secretory IgA in the intestinal
7 lumen.

8 One possible approach to overcoming these
9 problems is to homogeneously disperse the antigen of
10 interest within the polymeric matrix of appropriately
11 sized biodegradable, biocompatible microspheres that
12 are specifically taken up by GALT. Eldridge et. al.
13 have used a murine model to show that
14 orally-administered 1-10 micrometer microspheres
15 consisting of polymerized lactide and glycolide, (the
16 same materials used in resorbable sutures), were
17 readily taken up into Peyer's patches, and the 1-5
18 micrometer size were rapidly phagocytized by
19 macrophages. Microspheres that were 5-10 micrometers
20 (microns) remained in the Peyer's patch for up to 35
21 days, whereas those less than 5 micrometer disseminated
22 to the mesenteric lymph node (MLN) and spleen within
23 migrating MAC-1⁺ cells. Moreover, the levels of
24 specific serum and secretory antibody to staphylococcal
25 enterotoxin B toxoid and inactivated influenza A virus
26 were enhanced and remained elevated longer in animals

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1 which were immunized orally with microencapsulated
2 antigen as compared to animals which recieved equal
3 doses of non-encapsulated antigen. These data indicate
4 that microencapsulation of an antigen given orally may
5 enhance the mucosal immune response against enteric
6 pathogens. AF/R1 pili mediate the species-specific
7 binding of E. coli RDEC-1 with mucosal glycoproteins
8 in the small intestine of rabbits and are therefore an
9 important virulence factor. Although AF/R1 pili are
10 not essential for E. coli RDEC-1 to produce
11 enteropathogenic disease, expression of AF/R1 promotes
12 a more severe disease. Anti-AF/R1 antibodies have
13 been shown to inhibit the attachment of RDEC-1 to the
14 intestinal mucosa and prevent RDEC-1 disease in
15 rabbits. The amino acid sequence of the AF/R1 pilin
16 subunit has recently been determined, but specific
17 antigenic determinants within AF/R1 have not been
18 identified.

19 Recent advances in the understanding of B
20 cell and T cell epitopes have improved the ability to
21 select probably linear epitopes from the amino acid
22 sequence using theoretical criteria. B cell epitopes
23 are often composed of a string of hydrophilic amino
24 acids with a high flexibility index and a high
25 probability of turns within the peptide structure.
26 Prediction of T cell epitopes are based on the Rothbard

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1 method which identifies common sequence patterns that
2 are common to known T cell epitopes or the method of
3 Berzofsky and others which uses a correlation between
4 algorithms predicting amphipathic helices and T cell
5 epitopes.

6 In the current study we have used these
7 theoretical criteria to predict probable T or B cell
8 epitopes from the amino acid sequence of AF/R1. Four
9 different 16 amino acid peptides that include the
10 predicted epitopes have been synthesized: AF/R1 40-55
11 as a B cell epitope, 79-94 as a T cell epitope, 108-123
12 as a T and B cell epitope, and AF/R1 40-47/79-86 as a
13 hybrid of the first eight amino acids from the
14 predicted B cell epitope and the T cell epitope. We
15 have used these peptides as well as the native protein
16 to stimulate the in vitro proliferation of lymphocytes
17 taken from the Peyer's patch, MLN, and spleen of
18 rabbits which have recieved intraduodenal priming with
19 microencapsulated or non-encapsulated AF/R1. Our
20 results demonstrate the microencapsulation of AF/R1
21 potentiates the cellular immune response at the level
22 of the Peyer's patch, thus enhancing in vitro
23 lymphocyte proliferation to both the native protein and
24 its linear peptide antigens. CFA/I pili, rigid
25 thread-like structures which are composed of repeating
26 pilin subunits of 147 amino acid found on serogroups

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1 015, 025, 078, and 0128 of enterotoxigenic E. coli
2 (ETEC) [1-4, 18]. CFA/I promotes mannose resistant
3 attachment to human brush borders [5]; therefore, a
4 vaccine that established immunity against this protein
5 may prevent the attachment to host tissues and
6 subsequent disease. In addition, because the CFA/I
7 subunit shares N-terminal amino acid sequence homology
8 with CS1, CFA/II (CS2) and CFA/IV (CS4) [4], a subunit
9 vaccine which contained epitopes from this area of the
10 molecule may protect against infection with various
11 ETEC.

12 Until recently, experiments to identify these
13 epitopes were time consuming and costly; however,
14 technology is now available which allows one to
15 simultaneously identify all the T cell and B cell
16 epitopes in the protein of interest. Multiple Peptide
17 synthesis (Pepscan) is a technique for the simultaneous
18 synthesis of hundreds of peptides on polyethylene rods
19 [6]. We have used this method to synthesize all the
20 140 possible overlapping actapeptides of the CFA/I
21 protein. The peptides, still on the rods, can be used
22 directly in ELISA assays to map B cell epitopes [6,
23 12-14]. We have also synthesized all the 138 possible
24 overlapping decapeptides of the CFA/I protein. For
25 analysis of T cell epitopes, these peptides can be
26 cleaved from the rods and used in proliferation assays

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1 [15]. Thus this technology allows efficient mapping
2 and localization of both B cell and T cell epitopes to
3 a resolution of a single amino acid [16]. These
4 studies were designed to identify antigenic epitopes of
5 ETEC which may be employed in the construction of an
6 effective subunit vaccine.

7 CFA/I pili consist of repeating pilin protein
8 subunits found on several serogroups of enterotoxigenic
9 E. coli (ETEC) which promote attachment to human
10 intestinal mucosa. We wished to identify areas within
11 the CFA/I molecule that contain immunodominant T cell
12 epitopes that are capable of stimulating the
13 cell-mediated portion of the immune response in
14 primates as well as immunodominant B cell epitopes. To
15 do this, we (a) resolved the discrepancy in the
16 literature on the complete amino acid sequence of
17 CFA/I, (b) immunized three Rhesus monkeys with
18 multiple i.m. injections of purified CFA/I subunit in
19 Freund's adjuvant, (c) synthesized 138 overlapping
20 decapeptides which represented the entire CFA/I protein
21 using the Pepscan technique (Cambridge Research
22 Biochemicals), (d) tested each of the peptides for
23 their ability to stimulate the spleen cells from the
24 immunized monkeys in a proliferative assay (e)
25 synthesized 140 overlapping octapeptides which
26 represented the entire CFA/I protein, and (f) tested

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1 serum from each monkey for its ability to recognize the
2 octapeptides in a modified ELISA assay. A total of 39
3 different CFA/I decapeptides supported a significant
4 proliferative response with the majority of the
5 responses occurring within distinct regions of the
6 protein (peptides beginning with residues 8-40, 70-80,
7 and 127-137). Nineteen of the responsive peptides
8 contained a serine residue at positions 2, 3, or 4 in
9 the peptide, and a nine contained a serine specifically
10 at position 3. Most were predicted to be configured as
11 an alpha helix and have a high amphipathic index.
12 Eight B cell epitopes were identified at positions
13 3-11, 11-21, 22-29, 32-40, 38-45, 66-74, 93-101, and
14 124-136. The epitope at position 11-21 was strongly
15 recognized by all three individual monkeys, while the
16 epitopes at 93-101, 124-136, 66-74, and 22-29 were
17 recognized by two of the three monkeys.

18 V. SUMMARY OF THE INVENTION

19 This invention relates to a novel
20 pharmaceutical composition, a microcapsule/sphere
21 formulation, which comprises an antigen encapsulated
22 within a biodegradable polymeric matrix such as poly
23 (DL-lactide-co-glycolide) (DL-PLG), wherein the
24 relative ratio between the lactide and glycolide
25 component of the DL-PLG is within the range of 40:60 to
26 0:100, and its use, as a vaccine, in the effective

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1 pretreatment of animals (including humans) to prevent
2 intestinal infections caused by a virus or bacteria.
3 In the practice of this invention, applicants found
4 that the AF/R1 adherence factor is a plasmid encoded
5 pilus composed of repeating pilin protein subunits
6 that allows E. coli RDEC-1 to attach to rabbit
7 intestinal brush borders. To identify an approach that
8 enhances the immunogenicity of antigens that contact
9 the intestinal mucosa, applicants investigated the
10 effect of homogeneously dispersing AF/R1 pili within
11 biodegradable microspheres that included a size range
12 selected for Peyer's Patch localization. New Zealand
13 White rabbits were primed twice with 50 micrograms of
14 either microencapsulated or nonencapsulated AF/R1 by
15 endoscopic intraduodenal inoculation. Lymphoid tissues
16 were removed and cellular proliferative responses to
17 AF/R1 and synthetic AF/R1 peptides were measured in
18 vitro. The synthetic peptides represented possible T
19 and/or B cell epitopes which were selected from the
20 AF/R1 subunit sequence using theoretical criteria. In
21 rabbits which had received nonencapsulated AF/R1,
22 Peyer's Patch cells demonstrated slight but significant
23 proliferation in vitro in response to AF/R1 pili but
24 not the AF/R1 synthetic peptides. In rabbits which had
25 received microencapsulated AF/R1, Peyer's Patch cells
26 demonstrated a markedly enhanced response to AF/R1 and

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1 the synthetic peptides. Cells from the spleen and
2 mesenteric lymph nodes responded similarly to AF/R1
3 pili in both groups of animals, while there was a
4 greater response to the synthetic peptide AF/R1 40-55
5 in rabbits that had received microencapsulated AF/R1.
6 These data demonstrate that microencapsulation of AF/R1
7 potentiates the mucosal cellular immune response to
8 both the native protein and its linear peptide
9 antigens.

10 VI. BRIEF DESCRIPTION OF THE DRAWINGS

11 Figure 1 shows the size distribution of
12 microspheres wherein the particle size distribution (%)
13 is (a) By number 1-5 (91) and 6-10 (9) and (b) By
14 weight 1-5 (28) and 6-10 (72).

15 Figure 2 shows a scanning electron micrograph
16 of microspheres.

17 Figures 3(a) and (b) show the In vitro
18 immunization of spleen cells and demonstrates that
19 AF/RI pilus protein remains immunogenic to rabbit
20 spleen cells immunized in vitro after
21 microencapsulation. AF/R1 pilus protein has been found
22 to be immunogenic for rabbit spleen mononuclear cells
23 in vitro producing a primary IgM antibody response
24 specific to AF/RI. Immunization with antigen
25 encapsulated in biodegradable, biocompatible
26 microspheres consisting of lactide/glycolide copolymers

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1 has been shown to endow substantially enhanced immunity
2 over immunization with the free antigen. To determine
3 if microencapsulated AF/RI maintains the immunogenicity
4 of the free pilus protein, a primary in vitro
5 immunization assay was conducted. Rabbit spleen
6 mononuclear cells at a concentration of 3×10^5
7 cells/well. Triplicate wells of cells were immunized
8 with free AF/RI in a dose range from 15 to 150 ng/ml or
9 with equivalent doses of AF/RI contained in
10 microspheres. Supernatants were harvested on days 7,
11 9, 12, and 14 of culture and were assayed for free
12 AF/RI pilus protein specific IgM antibody by the ELISA.
13 Supernatant control values were subtracted from those
14 of the immunized cells. Cells immunized with free
15 pilus protein showed a significant positive IgM
16 response on all four days of harvest, with the antibody
17 response increasing on day 9, decreasing on day 12, and
18 increasing again on day 14. Cells immunized with
19 microencapsulated pilus protein showed a comparable
20 positive IgM antibody response as cells immunized with
21 free pilus protein. In conclusion, AF/RI maintains
22 immunogenicity to rabbit spleen cells immunized in
23 vitro after microencapsulation.

24 Figures 4(a) and (b) show in vitro
25 immunization of Peyer's patch cells. Here the AF/RI
26 pilus protein remains immunogenic to rabbit Peyer's

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1 patch cells immunized in vitro after
2 microencapsulation. AF/RI pilus protein has been found
3 to be immunogenic for rabbit Peyer's patch mononuclear
4 cells in vitro producing a primary IgM antibody
5 response specific to AF/RI. Immunization with antigen
6 encapsulated in biodegradable, biocompatible
7 microspheres consisting of lactide/glycolide copolymers
8 has been shown to endow substantially enhanced immunity
9 over immunization with the free antigen. To determine
10 if microencapsulated AF/RI maintains the immunogenicity
11 of the free pilus protein, a primary in vitro
12 immunization assay was conducted. Rabbit Peyer's patch
13 mononuclear cells at a concentration of 3×10^6 cells/ml
14 were cultured in 96-well, round bottom microculture
15 plates at a final concentration of 6×10^5 cells/well.
16 Triplicate wells of cells were immunized with free
17 AF/RI in a dose range from 15 to 150 ng/ml or with
18 equivalent dose of AF/RI contained in microspheres.
19 Supernatants were harvested on days 7, 9, 12, and 14 of
20 culture and were assayed for free AF/RI pilus protein
21 specific IgM antibody by the ELISA. Supernatant
22 control values were subtracted from those of the
23 immunized cells. Cells immunized with free pilus
24 protein showed a significant positive IgM response on
25 all four days of harvest, with the highest antibody
26 response on day 12 with the highest antigen dose.

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1 Cells immunized with encapsulated pilus protein showed
2 a positive response on day 12 with all three antigen
3 doses. In conclusion, AF/RI pilus protein maintains
4 immunogenicity to rabbit Peyer's patch cells immunized
5 in vitro after microencapsulation.

6 Figure 5 shows proliferative responses to
7 AF/RI by rabbit Peyer's patch cells. Naive rabbits
8 were primed twice with 50 micrograms of either
9 non-encapsulated (rabbits 132 and 133) or
10 microencapsulated (rabbits 134 and 135) AF/RI pili by
11 endoscopic intraduodenal inoculation seven days apart.
12 Seven days following the second priming, Peyer's patch
13 cells were cultured with AF/RI in 96-well plates for
14 four days followed by a terminal six hour pulse with
15 [³H]thymidine. Data shown is the SI calculated from
16 the mean cpm of quadruplicate cultures. Responses were
17 significant for all rabbits: 132 (p=0.013), 133
18 (p=.0006), 134 (p=0.0016), and 135 (p=0.0026).
19 Responses were significantly different between the two
20 groups. Comparison of the best responder in the
21 nonencapsulated antigen group (rabbit 133) with the
22 lowest responder in the microencapsulated antigen group
23 (rabbit 134) demonstrated an enhanced response when the
24 immunizing antigen was microencapsulated (p=0.0034).

25 Additionally, Figure 5 relates to the in
26 vitro lymphocyte proliferation after sensitization of

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1 rabbit lymphoid tissues with encapsulated or
2 non-encapsulated AF/RI pilus adhesion of E. coli strain
3 RDEC-1. The AF/RI adherence factor is a plasmid
4 encoded pilus protein that allows RDEC-1 to attach to
5 rabbit intestinal brush borders. We investigated the
6 immunopotentiating effect of encapsulating purified
7 AF/RI into biodegradable non-reactive microspheres
8 composed of polymerized lactide and glycolide,
9 materials used in resorbable sutures. The microspheres
10 had a size range of 5-10 microns, a size selected for
11 Peyer's Patch localization, and contained 0.62% protein
12 by weight. NZW rabbits were immunized twice with 50
13 micrograms of either encapsulated or non-encapsulated
14 AF/RI by intraduodenal later of non-encapsulated AF/RI
15 by intraduodenal inoculation seven days apart.
16 Lymphocyte proliferation in response to purified AR/RI
17 was conducted in vitro at seven days and showed that
18 encapsulating the antigen into microspheres enhanced
19 the cellular immune response in the Peyer's Patch;
20 however, no significant increase was observed in spleen
21 or mesenteric lymph node. These data suggest that
22 encapsulation of AF/RI may potentiate the mucosal
23 cellular immune response.

24 Figures 6 a-d show proliferative responses to
25 AF/RI synthetic peptides by rabbit Peyer's patch
26 cells. Naive rabbits were primed twice with 50

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1 micrograms of either non-encapsulated (rabbits 132 and
2 133) or microencapsulated (rabbits 134 and 135) AF/RI
3 pili by endoscopic intraduodenal inoculation seven days
4 apart. Seven days following the second priming,
5 Peyer's patch cells from each rabbit were cultured with
6 AF/RI 40-55 (Fig. 6a), AF/RI 79-94 (Fig. 6b), AF/RI
7 108-123 (Fig 6c), or AF/RI 40-47/79-86 (Fig. 6d) in
8 96-well plates for four days followed by a terminal six
9 hour pulse with [³H]thymidine. Data shown is the SI
10 calculated from the mean cpm of quadruplicate cultures.
11 The responses of rabbits 132 and 133 were not
12 significant to any of the peptides tested. Rabbit 134
13 had a significant response to (a) AF/RI 40-55
14 (p=0.0001), (b) AF/RI 79-94 (p=0.0280), and (d) AF/RI
15 40-57/79-86 (p=0.025), but not to (c) AF/RI 108-123.
16 Rabbit 135 had a significant response to (a) AF/RI
17 40-55 (p=0.034), (b) AF/RI 79-94 (p=0.040), and (c)
18 AF/RI 108-123 (p<0.0001), but not to (d) AF/RI
19 40-47/79-86. This demonstrates enhanced proliferative
20 response to peptide antigens following mucosal priming
21 with microencapsulated pili. AF/RI pili promotes
22 RDEC-1 attachment to rabbit intestinal brush borders.
23 Three 16 amino acid peptides were selected by
24 theoretical criteria from the AF/RI sequence as
25 probable T or B cell epitopes and were synthesized:
26 AF/RI 40-55 as a B cell epitope, 79-94 as a T cell

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1 epitope, and 108-123 as a T and B cell epitope. We
2 used these peptides to investigate a possible
3 immunopotentiating effect of encapsulating purified
4 Af/RI pili into biodegradable, biocompatible
5 microspheres composed of polymerized lactide and
6 glycolide at a size range that promotes localization in
7 the Peyer's Patch (5-10 micrometers). NZW rabbits
8 were primed twice with 50 micrograms AF/RI by
9 endoscopic intraduodenal inoculation and their Peyer's
10 Patch cells were cultured in vitro with the AF/RI
11 peptides. In two rabbits which had received
12 encapsulated AF/RI, lymphocyte proliferation was
13 observed to AF/RI 40-55 and 79-94 in both rabbits and
14 to 108-123 in one of two rabbits. No responses to any
15 of the peptides were observed in rabbits which received
16 non-encapsulated AF/RI. These data suggest that
17 encapsulation of AF/RI may enhance the cellular
18 response to peptide antigens.

19 Figures 7a-d show B-cell responses of Peyer's
20 patch cells to AF/R1 and peptides.

21 Figures 8a-d show B-cell responses of Peyer's
22 Patch cells to AF/R1 and peptides.

23 Figures 9a-d show B-cell responses of spleen
24 cells to AF/R1 and Peptides.

25 Figures 10a-d show B cell responses of spleen
26 cells to AF/R1 and peptides.

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1 Figures 7 through 10, illustrate enhanced
2 lymphocyte antibody response by mucosal immunization of
3 rabbits with microencapsulated AF/R1 pilus protein.
4 The AF/R1 pilus protein has been found to be
5 immunogenic for rabbit spleen and Peyer's patch cells
6 in vitro producing a primary IgM antibody response.
7 The purpose of this study was to determine if AR/R1
8 pilus protein immune response is enhanced by
9 microencapsulation. The AF/R1 was incorporated into
10 biodegradable, biocompatible microspheres composed of
11 lactide-glycolide copolymers, had a size range of 5-10
12 micrometer and containing 0.62% pilus protein by
13 weight. Initially, NZW rabbits were immunized twice
14 with 50 micrograms of either encapsulated or
15 non-encapsulated AF/R1 via intraduodenal route seven
16 days apart. For in vitro challenge, 6×10^5 rabbit
17 lymphocytes, were set in microculture at final volume
18 of 0.2 ml. Cells were challenged with AR/R1 or three
19 different synthetic 16 amino acid peptides
20 representing, either predicted T, B or T and B cell
21 epitopes in a dose range of 15 to 150 ng/ml for splenic
22 cells or 0.05 to 5.0 micrograms/ml for Peyer's patch
23 mononuclear cells (in triplicate). Supernatants were
24 collected on culture days 3, 5, 7, and 9 assayed by
25 ELISA for anti-AF/R1 antibody response as compared to
26 cell supernatant control. Significant antibody

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1 responses were seen only from spleen and Peyer's patch
2 cells from rabbits immunized with microencapsulated
3 AF/R1. The antibody response tended to peak between
4 days 5 and 9 was mainly an IgM response. The results
5 for the predicted epitopes were similar to those
6 obtained with purified AF/R1. In conclusion,
7 intestinal immunization with AF/R1 pilus protein
8 contained within microspheres greatly enhances both the
9 spleen and Peyer's patch B-cell responses to predicted
10 T & B-cell epitopes.

11 Figure 11 shows proliferative responses to
12 AF/R1 40-55 by rabbit MLN cells. Naive rabbits were
13 primed twice with 50 micrograms of either
14 nonencapsulated (rabbits 132 and 133) or
15 microencapsulated (rabbits 134 and 135) AF/R1 pili by
16 endoscopic intraduodenal inoculation seven days apart.
17 Seven days following the second priming, MLN cells were
18 cultured with AF/R1 40-55 for four days in 24-well
19 plates. Cultures were transferred into 96-well plates
20 for a terminal [³H]thymidine pulse. Data shown is the
21 SI calculated from the mean cpm of quadruplicate
22 cultures. Responses of rabbits 132 and 133 were not
23 statistically significant. Responses were significant
24 for rabbits 134 ($p=0.0051$) and 135 ($p=0.0055$).

25 Figure 12 shows proliferative responses to
26 AF/R1 40-55 by rabbit spleen cells. Naive rabbits were

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1 primed twice with 50 micrograms of either
2 nonencapsulated (rabbits 132 and 133) or
3 microencapsulated (rabbits 134 and 135) AF/R1 pili by
4 endoscopic intraduodenal inoculation seven days apart.
5 Seven days following the second priming, spleen cells
6 were cultured with AF/R1 40-55 for four days in 24-well
7 plates. Cultures were transferred into 96 well plates
8 for a terminal [³H]thymidine pulse. Data shown is the
9 SI calculated from the mean cpm of quadruplicate
10 cultures. Responses of rabbits 132 and 133 were not
11 statistically significant. Responses were significant
12 for rabbits 134 (p=0.0.0005) and 135 (p=0.0066).

13 Figure 16. A. SDS-PAGE of intact CFA/I (lane
14 1), trypsin treated CFA/I (lane 2), and *S. aureus* V8
15 protease treated CFA/I. Molecular masses of individual
16 bands were estimated from molecular weight standards
17 (on left). Multiple lanes of both trypsin and V8
18 treated CFA/I were transferred to PVDF membranes where
19 bands corresponding to the approximate molecular masses
20 of 3500 (trypsin digest, see arrow lane 2) and 6000 (V8
21 digest, see arrow lane 3) were excised and subjected to
22 Edman degradation. B. Resulting sequence of protein
23 fragments from each lane of A (position of sequenced
24 portion of fragment in the intact protein. Underlined,
25 italicized residues are amino acids under dispute in
26 literature.

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1 Figure 17. ELISA assay results testing
2 hyperimmune sera of monkeys (A)2Z2 (monkey 3), (B)
3 184(D) (monkey 1) and (C) 34 (monkey 2) to CFA/I
4 primary structure immobilized on polyethylene pins.
5 Monkey sera diluted 1:1000. Peptide number refers
6 first amino acid in sequence of octapeptide on pin from
7 CFA/I primary structure OD 405 refers to optical
8 density wavelength at which ELISA plates were read (405
9 nm).

10 Figure 18. Complete sequence of CFA/I (147
11 amino acids) with B cell recognition site (boxed areas)
12 as defined by each individual monkey response (2Z2,
13 184D, and 34). Derived from data in Figure 17.

14 Figures 19-21. Lymphocyte proliferation to
15 synthetic decapeptides of CFA/I. Each monkey was
16 immunized with three i.m. injections of CFA/I subunits
17 in adjuvant, and its spleen cells were cultured with
18 synthetic decapeptides which had been constructed using
19 the Pepsan technique. The decapeptides represented
20 the entire CFA/I protein. Concentrations of synthetic
21 peptide used included 6.0, 0.6, and 0.06 micrograms/ml.
22 Values shown represent the maximum proliferative
23 response produced by any of the three concentrations of
24 antigen used \pm the standard deviation. The cpm of the
25 control peptide for each of the three monkeys was 1,518
26 \pm 50, 931 \pm 28, and 1,553 \pm 33 respectively. The cpm

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1 of the media control for each of the three monkeys was
2 $1,319 \pm 60$, 325 ± 13 , and $1,951 \pm 245$ respectively.

3 Figures 22-24. Lymphocyte proliferation to
4 6.0, 0.6, and 0.06 micrograms/ml synthetic decapeptides
5 of CFA/I in one monkey. The monkey (2Z2) as immunized
6 with three i.m. injections of CFA/I subunits in
7 adjuvant, and its spleen cells were cultured with
8 synthetic decapeptides which had been constructed using
9 the Pepsan technique. The decapeptides represented
10 the entire CFA/I protein. Values shown represent the
11 proliferative response which occurred to 6.0
12 micrograms/ml (Fig. 22), 0.6 micrograms/ml (Fig. 23),
13 or 0.06 micrograms/ml (Fig. 24) of antigen \pm the
14 standard deviation. The cpm of the control peptide was
15 $1,553 \pm 33$ and the cpm of the media control was $1,951 \pm$
16 245.

17 Figure 25 shows that rabbits numbers 21 and
18 22 received intraduodual administration of AF/R1
19 microspheres at doses of AF/R1 of 200 micrograms (ug)
20 on day 0 and 100 ug on day 7, 14, and 21 then
21 sacrificed on day 31. The spleen, Peyer's patch and
22 ileal lamina propria cells at 6×10^5 in 0.2 ml in
23 quadriplate were challenged with AF/RI and AF/R1 1-13,
24 40-55, 79-94, 108-123, and 40-47, 79-85 synthetic
25 peptides at 15, 1.5 and .15 ug/ml for 4 days. The
26 supernatants were tested for IL-4 using the IL-4/IL-2

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1 dependent cell line cells CT4R at 50,000/well with 0.1
2 ml of 6.25% supernatant for 3 days then pulsed with
3 tritiated thymidine for 4 hrs, cells harvested and the
4 tritiated thymidine incorporation determined, averaged
5 and expressed with one standard deviation thousand
6 counts per minute (kcpm).

7 Figure 26 shows that RDEC-1 colonization (log
8 CFU/gm) in cecal fluids was similar in both groups
9 (mean 6.3 vs 7.3; $p=.09$).

10 Figure 27 shows that rabbits given AF/R1-MS
11 remained well and 4/6 gained weight after challenge,
12 whereas 9/9 unvaccinated rabbits lost weight after
13 challenge (mean weight change +10 vs -270 grams
14 $p<.001$).

15 Figure 28 shows that the mean score of RDEC-1
16 attachment to the cecal epithelium was zero in
17 vaccinated, and 2+ in unvaccinated animals.

18 Figure 29. Particle size distribution of
19 CFA/II microsphere vaccine Lot L74F2 values are percent
20 frequency of number or volume verses distribution.
21 Particle size (diameter) in microns. 63% by volume are
22 between 5-10 um and 88% by volume are less then 10 um.

23 Figure 30. Scanning electron photomicrograph
24 of CFA/II microsphere vaccine Lot L7472 standard bar
25 represents 5 um distance.

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1 Figure 31. Twenty-two hour CFA/II release
2 study of CFA/II microsphere vaccine Lot L7472. Percent
3 cumulative release of CFA/II from three sample: A,
4 33.12 mgm; B, 29.50 mgm c, 24.20 mgm at 1, 3, 6, 8, 12
5 and 22 hour intervals. Average represents the mean \pm
6 ISD.

7 Figure 32. Serum IgG antibody reponse to
8 CFA/II microsphere vaccine Lot L7472 following 2 25 ug
9 protein IM immunization on day 0 in 2 rabbits.
10 Antibody determines on serial dilution of sera by ELISA
11 and expressed as mean titer versus day 0, 7 and 14.

12 Figure 33. Serum IgG antibody response to
13 CFA/II microsphere vaccine Lot L7F2 following 2 25 ug
14 protein IM immunizations on day 0 if rabbit 107 & 109.
15 Antibody determined on serial dilution (in duplicate)
16 of sera by ELISA and expressed as mean titer versus day
17 0, 7 and 14.

18 Figure 34. Lymphocyte proliferative
19 responses for Peyer's patch cells of rabbits 65 (figure
20 34 (a)), 66 (figure 34 (b)) , 83 (figure 34 (c)), 86
21 (figure 34 (d)), and 87 (figure 34 (e)) immunized
22 intraduodenally with 50 mgm protein of CFA/II
23 microsphere vaccine 4 and 7 days earlier. The cells
24 are challenged in vitro with CFA/II or BSA at 500, 50
25 and 5 ug/ml or media in triplicate. The uptake of
26 tritiated thymidine in Kcp is expressed as mean \pm ISD.

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1 Using the paired student t-test, the p values of 500
2 ug/ml dose of CFA/II compared to media control are:
3 65, $p = 0.0002$; 66, $p = 0.0002$; 83, $p = 0.0002$; and 86, p
4 $= 0.0002$.

5 Figure 35. Lymphocyte proliferative
6 responses from Peyer's patch cells of rabbits 77
7 (figure 35 (a)), 78 (figure 35 (b)), 80 (figure 35
8 (c)), 88 (figure 35 (d)), and 91 (figure 35 (e))
9 immunized intraduodenally with 50 mgm protein of CFA/II
10 microspheres vaccine 14 and 7 days earlier. The cells
11 are challenged in vitro with CFA with CFA/II or BSA at
12 500, 50 and 5 ug/ml or media in triplicate the uptake
13 of trisplate. The uptake of tritiated thymidine in
14 Kcp is expressed as mean \pm ISD. Using the paired
15 student t -test, the protein of 500 ug/ml dose of
16 CFA/II compared to media control are: 77, $p = 0.0001$;
17 78; $= 0.0015$; 80, $p =$ insignificant; 88, $p = 0.0093$;
18 and 91 $p = 0.0001$.

19 Figure 36. ELISPOT assay of spleen cells
20 from rabbits 65 (figure 36 (a)), 66 (figure 36 (b)), 83
21 (figure 36 (c)), 86 (figure 36 (d)), and 87 (figure 36
22 (e)) immunized intraduodenally with 50 mgm protein
23 of CFA/II microsphere vaccine 14 and 7 days
24 earlier. These were cells placed into microculture and
25 tested on day 0, 1, 2, 3, 4 and 5 by ELISPOT for cells
26 secreting antibodies specific for CFA/II antigen. The

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1 results are expressed as number per 9×10^6 spleen
2 cells versus culture day tested.

3 Figure 37. ELISPOT assay of spleen cells
4 from normal control rabbits, 67, 69, 72 and 89. The
5 cells were placed into microculture and tested on days
6 0, 1, 2, 3, 4 and 5 by ELISPOT for cells secreting
7 antibodies specific for CFA/II antigen. The results
8 are expressed as number per 9×10^6 spleen cells versus
9 culture day tested.

10 Figure 38. Curve for determining vaccination
11 dosages for regimen b.

12 Figure 39. Hepatitis B surface antigen
13 release from 50:50 poly (DL-lactide-co-glycolide).

14 Figures 11 and 12 serve to illustrate that
15 inclusion of Escherichia coli pilus antigen in
16 microspheres enhances cellular immunogenicity.

17 A primary mucosal immune response,
18 characterized by antipilus IgA, follows infection of
19 rabbits with E. coli RDEC-1. However, induction of an
20 optimal primary mucosal response by enteral vaccination
21 with pilus antigen depends on immunogenicity of pilus
22 protein, as well as such factors as its ability to
23 survive gastrointestinal tract (GI) transit and to
24 target immunoresponsive tissue. We tested the effect
25 of incorporating AF/R1 pilus antigen into resorbable
26 microspheres upon its ability to induce primary mucosal

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1 and systemic antibody responses after direct
 2 inoculation into the GI tract. METHODS: rabbits were
 3 inoculated with 50 micrograms of AF/R1 pilus antigen
 4 alone or incorporated into uniformly sized (5-10
 5 microns) resorbably microspheres (MIC) of
 6 poly(DL-lactide-coglycolide). Inoculation was by
 7 intra-duodenal (ID) intubation via endoscopy or
 8 directly into the ileum near a Peyer's patch via the
 9 RITARD procedure (with the cecum ligated to enhance
 10 recovery of gut secretions and a reversible ileal tie
 11 to slow antigen clearance). ID rabbits were sacrificed
 12 at 2 weeks for collection of gut washes and serum.
 13 RITARD rabbits were bled and purged weekly for 3 weeks
 14 with Co-lyte to obtain gut secretions. Anti-pilus IgA
 15 and IgG were measured by ELISA.

TABLE 1

17	<u>RESULTS</u> :*pos/test	RITARD-PILI	RITARD-MIC	ID-PILI
18	ID-MIC			
19	Anti-pilus IgA (fluid)	*7/8		4/8
20	1/2 0/3			
21	Anti-pilus IgG (serum)	0/8		3/8
22	0/2 1/3			

23 Native pilus antigen led to a mucosal IgA
 24 resposne in 7/8 RITARD rabbits. MIC caused a similar
 25 response in only 4/8, but the groups were not
 26 statistically different. MIC (but not pili) induced

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1 some systemic IgG responses (highest in animals without
2 mucosal responses). Results in rabbits inoculated ID
3 were similar for pili, but no mucosal response to
4 ID-MIC was noted. SUMMARY: Inoculation with pilus
5 antigen produces a primary mucosal IgA response.
6 Microencapsulation does not enhance this response,
7 although the antigen remains immunogenic as shown by
8 measurable mucosal and some strong serum responses. It
9 must be determined whether priming with antigen in
10 microspheres can enhance secondary responses.

11 B CELL EPITOPE DATA

12 Materials and Methods

13 **CFA/I PURIFICATION-** INTACT CFA/I pili were
14 purified from H10407 (078:H-) as described by Hall et
15 al, (1989) [20]. Briefly, bacteria grown on
16 colonization factor antigen agar were subjected to
17 shearing, with the shearate subjected to differential
18 centrifugation and isopycnic banding on cesium chloride
19 in the presence of N-lauryl sarkosine. CFA/I were
20 dissociated to free subunits in 6M guanididinium HCl,
21 0.2 M ammonium bicarbonate (2 hr, 25°), passed through
22 an ultrafiltration membrane (Amicon XM 50 stirred cell,
23 Danvers, MA), with concentration and buffer exchange to
24 PBS on a YM 10 stirred cell (Amicon). Examination of
25 dissociated pili by electron microscopy demonstrated a
26 lack of pilus structure.

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1 **Protein Sequencing-** The primary structure of
2 CFA/I has been determined by protein sequencing
3 techniques (Klemm, 1982) and through molecular cloning
4 methods (Karjalainen, et al 1989) [21]. In these two
5 studies there was agreement in all but two of the 147
6 amino acid residues (at positions 53 and 74). To
7 resolve the apparent discrepancies, CFA/I was
8 enzymatically digested in order to obtain internal
9 amino acid sequence. Trypsin or *S. aureus* V8 protease
10 (sequencing grade, Boehringer Mannheim) was incubated
11 with CFA/I at a 1:50 w:w ratio (Tris 50 mM, 0.1% SDS,
12 pH 8.5 for 16h at 37° (trypsin) or 24°C (V8)). Digested
13 material was loaded onto precast 16% tricine SDS-PAGE
14 gels (Schagger and von Jagow, 1987) (Novex, Encinitis,
15 CA) and run following manufacturers instructions.
16 Separated samples were electrophoretically transferred
17 to PVDF membranes (Westrans, Schleicher and Schuell,
18 Keene, NH) following Matsiduria (1987) using the Novex
19 miniblot apparatus. Blotted proteins were stained with
20 Rapid Coomassie stain (Diversified Biotech, Newton
21 Centre, MA). To obtain the desired fragment containing
22 the residue of interest within a region accessible by
23 automated gas phase sequencing techniques, molecular
24 weights were estimated from standards of molecular
25 weights 20,400 to 2,512 (trypsin inhibitor, myoglobin,
26 and myoglobin cyanogen bromide fragments; Diversified

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1 Biotech) using the corrected molecular weights for the
2 myoglobin fragments as given in Kratzin et al., (1989)
3 [22]. The estimated molecular weights for the unknown
4 CFA/I fragments were compared to calculated molecular
5 weights of fragments as predicted for CFA/I from the
6 sequence of CFA/I as analysed by the PEPTIDESORT
7 program of a package developed by the University of
8 Wisconsin Genetics Computer Group. Selected fragments
9 were cut from the PVDF emembrane and subjected to gas
10 phase sequencing (Applied Biosystem 470, Foster City,
11 CA).

12 Monkey Immunization- Three rhesus monkeys (Macaca
13 mulatta) were injected intramuscularly with 250 ug of
14 dissociated CFA/I in complete Freund's adjuvant and
15 subsequently with two injections of 250 ug of antigen in
16 incomplete Freund's adjuvant at weekly intervals.
17 Blood was drawn three weeks after primary immunization.

18 Peptide Synthesis- Continuous overlapping
19 octapeptides spanning the entire sequence CFA/I were
20 synthesized onto polyethylene pins by the method of
21 Geysen et al. [16], also known as the PEPSCAN
22 procedure. Derivitized pins and software were
23 purchased from Cambridge Research Biochemicals (Valley
24 Stream, NY). Fmoc-amino acid pentafluorophenyl esters
25 were purchased from Peninsular Laboratories (Belmont,
26 CA), 1-hydroxybenzotriazole monohydrate (HYBT) was

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1 purchased from Aldrich, and reagent grade solvents from
2 Fisher. To span the entire sequence of CFA/I with a
3 single amino acid overlap of from one peptide to the
4 next, 140 total pins were necessary, with a second
5 complete set of 140 pins synthesized simultaneously.

6 ELISA procedure- Sera raised in monkeys to
7 purified dissociated pili were incubated with the pins
8 in the capture ELISA assay of Geysen et al., [16] with
9 the preimmune sera of the same animal tested at the
10 same dilution simultaneously with the duplicate set of
11 pins. Dilution of sera used on the pins was chosen by
12 initial titration of sera by standard ELISA assay and
13 immunodot blot assay against the same antigen.

14 RESULTS

15 It was essential to utilize the correct sequence
16 of CFA/I in the synthesis of the pins for both T- and
17 B-cell experiments to carry out the studies as planned.
18 At issue were the amino acids at position 53 and 74;
19 incorrect residues at those positions would effect 36
20 of 138 pins (26%) for T-cell epitope analysis and 30 of
21 140 pins (21%) for B-cell analysis. To resolve the
22 discrepancy in the literature, purified CFA/I was
23 proteolytically digested separately with trypsin and
24 with *S. aureus* V8 protease (V8). These enzymes were
25 chosen in order to give fragments with the residues of
26 interest (53 and 74) relatively near to the N-terminus

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1 for automated Edman degradation (preferably 1-15
2 residues). These digests were separated on tricine
3 SDS-PAGE gels (Fig. 16A) and molecular masses of
4 fragments estimated. A fragment of 3459 calculated
5 molecular mass is expected from the trypsin digest
6 (corresponding to amino acids 62-94) and a fragment of
7 5889 calculated molecular mass is expected from the V8
8 digest (residues 42-95). These fragments were located
9 within each digest (arrows in Fig. 16), and a companion
10 gel with four lanes of each digest was run,
11 electrophoretically transferred to PVDF, the bands
12 excised and sequenced. N-terminal sequences of each
13 fragment are given in Fig. 16B. The N-terminal
14 eighteen residues from the trypsin fragment were
15 determined that corresponded to positions 62-79 in
16 CFA/I. Position 74, a serine residue was consistent
17 with that determined by Karjalainen et al.,
18 (Karjalainen et al., 1989). Nineteen residues of the
19 V8 fragment were determined, corresponding to residues
20 41-60 of the parent protein. The twelfth residue of the
21 fragment contained an aspartic acid, also consistent
22 with Karjalainen et al., (1989). All other residues
23 sequenced were consistent with those published
24 previously (including residues 1-29, not shown). For
25 the following peptide synthesis were therefore

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1 utilized the complete amino acid sequence of CFA/I
2 consistent with Karjalainen et al., (1989).

3 Sera from monkeys immunized with CFA/I subunits
4 were tested in a modified ELISA assay, with the
5 preimmunization sera tested simultaneously with
6 duplicate pins. Assays results are displayed in Fig.
7 17. Monkey 2Z2 (fig. 2A) responded strongly to six
8 regions of the CFA/I sequence. Peptide 14 (the
9 octapeptide 14-21) gave the strongest response with
10 four pins adjacent to it (11, 12, 13, and 15) also
11 appearing to bind significant antibody. The other 2Z2
12 epitopes are centered at peptides 3, 22, 33, 93, and
13 124. Monkey 184D (Fig. 17B) also responded strongly to
14 peptide 14, although the maximum response was to
15 peptide 13, with strong involvement of peptide 12 in
16 the epitope. Additional epitopes recognized by 184D
17 were centered at peptides 22, 33, 66, and 93. The
18 third monkey serum tested, 34, responded to this region
19 of the CFA/I primary structure, both at peptides 1, 12
20 and weakly at 14. Two other epitopes were identified
21 by 34, centered at peptides 67 and 128. Figure 18
22 illustrates the amino acids corresponding to the
23 epitopes of CFA/I as defined by the response of these
24 three monkeys aligned with the entire primary
25 structure. The entire antigenic determinants are
26 mapped and areas of overlap with other epitopes

-33-

1 (consensus sites) are displayed. These epitopes are
2 summarized in Table 1.

3 T Cell Epitope Data

4 Materials and Methods

5 Animals. Three healthy adult Macaca mulatta
6 (Rehesus) monkeys (approximately 7 kg each) were used
7 in this study. Their medical records were examined to
8 assure that they had not been in a previous protocol
9 which would preclude their use in this study. Each
10 monkey was sedated with ketamine HCL1 at standard
11 dosage and blood was drawn to obtain preimmune serum.

12 Antigen. CFA/I pili were purified from E. coli
13 strain H107407 (serotype 078:H11) by ammonium sulfate
14 precipitation using the method of Isaacson [17]. The
15 final preparation migrated as a single band on
16 SD-polyacrylamide gel electrophoresis and was shown to
17 be greater than 95% pure by scanning with laser
18 desitometry when stained with coomassie blue. The pili
19 were then dissociated into CFA/I pilin subunits.

20 Immunization. Each monkey was given 25 mg of
21 purified CFA/I pilin subunits, which had been
22 emulsified in Complete Freund's Adjuvant, by single
23 i.m. injection (0.5 ml). For each animal, the initial
24 dose of antigen was followed by two similar injections
25 in Incomplete Freund's Adjuvant at seven day intervals.

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1 Peptide Antigens. The peptides were synthesized
2 based on the published sequence of CFA/I [18] using the
3 Geysen pin method (Pepscan procedure) [16] with
4 equipment and software purchased from Cambridge
5 Research Biochemicals, Inc. (Wilmington, DE).
6 Fmoc-amino acid pentafluorophenyl esters were purchased
7 from Peninsula Laboratories (Belmont, CA) and used
8 without further treatment or analysis. The activating
9 agent 1-hydroxybenzotriazole monohydrate (HOBT) was
10 purchased from Aldrich Chemical Company (Milwaukee,
11 WI). Solvents were reagent grade from Fisher Scientific
12 (Springfield, NJ).

13 Two schemes were used to synthesize the peptides.
14 Peptides for the B-cell tests were synthesized as
15 octamers and remained linked to the resin. However,
16 the peptides used to search for T-cell epitopes were
17 synthesized as decamers with an additional Asp-Pro
18 spacer between the pins and the peptides of interest.
19 The Asp-Pro linkage is acid labile allowing cleavage of
20 the decamers from the pins for T-cell proliferation
21 assays [15]. The peptides were cleaved in 70% formic
22 acid for 72 hours at 37 degrees C. The acid solution
23 was removed by evaporation (Speed-Vac, Savant
24 Instruments, Framingdale, NY) followed by rehydration
25 with distilled deionized water and lyophilizaiton. The
26 resulting cleaved peptides were used without further

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1 treatment or analysis. The yield was approximately 10
2 ug per pin, approximately 10 per cent on a molar basis
3 of the total amount of proline on each pin as
4 determined by quantitative amino acid analysis.

5 Residues 12 and 13 on the CFA-1 protein are Asp
6 and Pro, respectively, the same sequence used to cleave
7 the peptides from the pins. Therefore, to prevent
8 truncated peptides from the native sequence during the
9 cleavage process, two substitutions were made for
10 Asp-12. One substitution was a glutamic acid residue
11 for the aspartic acid, a substitution to retain the
12 carboxylic acid functional group. The second
13 substitution was an asparagine residue to conserve the
14 approximate size of the side chain while retaining some
15 hydrophilicity. Each substitution was tested in the
16 T-cell proliferation assay. Both substitutions as well
17 as the native sequence were analyzed by ELISA. For
18 both the T cell and B cell assays, additional sequences
19 not found on the protein were synthesied and used as
20 control peptides.

21 Lymphocyte proliferation. At day 10-14 following
22 the final inoculation of antigen, the monkeys were
23 again sedated with ketamine HCl, and 50 ml of blood was
24 drawn from the femoral artery for serum preparation.
25 Animals were then euthanized with an overdose of
26 pentothal and spleen was removed. Single cell

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1 suspensions were prepared and washed in Dulbecco's
2 modified Eagle medium (Gibco Laboratories, Grand
3 Island, NY) which had been supplemented with penicillin
4 (100 units/ml), streptomycin (100 ug/ml), L-glutamine
5 (2mM), and HEPES Buffer (10 mM) all obtained from Gibco
6 Laboratories, as well as MEM non-essential amino acid
7 solution (0.1 mM), MEM [50x] amino acids (2%), sodium
8 bicarbonate (0.06%), and 5×10^{-5} M 2-ME all obtained
9 from Sigma Chemical Company (St. Louis, MO) [cDMEM].
10 Erythrocytes in the spleen cell suspension were lysed
11 using standard procedures in an ammonium chloride
12 lysing buffer. Cell suspensions were adjusted to 10^7
13 cells per ml in cDMEM, and autologous serum was added to
14 yield a final concentration of 1.0%. Cells (0.05 ml)
15 were plated in 96-well flat bottom culture plates
16 (Costar, Cambridge, MA) along with 0.05 ml of various
17 dilutions of antigen in cDMEM without serum (yielding a
18 0.5% final concentration of autologous serum) and were
19 incubated at 37 degrees C in 5% CO₂. Each peptide was
20 tested at 6.0, 0.6, 0.06 ug/ml. All cultures were
21 pulsed with 1 uci [³H]thymidine (25 Ci/mmol, Amersham,
22 Arlington Heights, IL) on day 4 of culture and were
23 harvested for scintillation counting 6 hours later.

ELISA.

25 Epitope prediction. Software designed to predict
26 B cell epitopes based on hydrophilicity, flexibility,

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1 and other criteria was developed by the University of
2 Wisconsin Genetics Computer Group [19]. Software
3 designed to predict T cell epitopes based on the
4 Rothbard method [7] was written by Stephen Van Albert
5 (The Walter Reed Army Institute of Research,
6 Washington, D.C.). Software designed to predict T cell
7 epitopes based on the Berzofsky method was published as
8 the AMPHI program [9]. It predicts amphipathic amino
9 acid segments by evaluating 7 or 11 residues as a block
10 and assigning the score to the middle residue of that
11 block.

12 Statistics. All lymphocyte proliferations were
13 conducted in replicates of four, and standard
14 deviations of the counts per minute (cpm) are shown.
15 Statistical significance (p value) for the
16 proliferative assay was determined using the Student's
17 t test to compare the cpm of quadruplicate wells
18 cultured with the CFA/I peptides to the cpm of wells
19 cultured with a control peptide.

20 RESULTS

21 Prediction of T cell epitopes within the CFA/I
22 molecule. To identify possible T cell epitopes within
23 the CFA/I molecule, amphipathic amino acid segments
24 were predicted by evaluating 7 or 11 residues as a
25 block using the AMPHI program [9]. Possible t cell
26 epitopes were also identified using criteria published

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1 by Rothbard and Taylor [7]. The sequence numbers of
2 the first amino acid of the predicted segments are
3 shown in Table 1.

4 Lymphocyte proliferation of monkey spleen cells
5 to CFA/I synthetic peptides. To determine which
6 segments of the CFA/I protein are able to stimulate
7 proliferation of CFA/I immune primate lymphocytes in
8 vitro, three Rhesus monkeys were immunized with CFA/I
9 subunits, and their splenic lymphocytes were cultured
10 with synthetic overlapping decapeptides which
11 represented the entire CF/I sequence. Concentrations
12 of peptides used as antigen were 6.0, 0.6, and 0.6
13 ug/ml. Proliferative responses to the decapeptides
14 were observed in each of the three monkeys (fig. 1-3).
15 The majority of the responses occurred at the 0.6 and
16 0.06 ug/ml concentrations of antigen and within
17 distinct regions of the protein (peptides beginning
18 with residues 8-40, 70-80, and 27-137). A comparison
19 of the responses at the 6.0, 0.6 and 0.06 ug/ml
20 concentrations antigenic peptide for one monkey (2&2)
21 are shown (fig. 4-6). Taking into account all
22 concentrations of antigen tested, spleen cells from
23 monkey 184D demonstrated a statistically significant
24 response to decapeptides beginning with CFA/I amino
25 acid residues 3, 4, 8, 12, 15, 21, 26, 28, 33, 88, 102,
26 10, 133, 134, and 136 (fig. 19). Monkey 34 had a

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1 significant response to decapeptides beginning with
2 residues 24, 31, 40, 48, 71, 72, 77, 78, 80, 87, and
3 102, 126 and 133 (Fig. 20); monkey 2Z2 responded to
4 decapeptides which began with residues 4, 9, 11, 12,
5 13, 14, 15, 16, 17, 20, 27, 35, 73, 79, 18, 127, 129,
6 132, and 133 (fig. 19). Peptides beginning with amino
7 acid residues 3 through 2 were synthesized with either
8 a glutamic acid or an asparagine substituted for the
9 aspartic acid residue at position twelve to prevent
10 truncated peptides. The observed responses to peptides
11 beginning with residue 8 (monkey 184d), and residues 9,
12 11, 12 (monkey 2Z2) occurred in response to peptides
13 that had the glutamic acid substitution. However, the
14 observed responses to peptides beginning with residue
15 3, 4, and 12 (monkey 184D), as well as residue 4 (monkey
16 2Z2) occurred in response to peptides that had the
17 asparagine substitution. Monkey 34 did not respond to
18 any of the peptides that had the substitution at
19 position twelve. All other responses shown were to the
20 natural amino acid sequence of the CFA/I protein.
21 Statistical significance was determined by comparing
22 the cpm of quadruplicate wells cultured with the CFA/I
23 peptides to the cpm of wells cultured with the CFA/I
24 peptides to the cpm of wells cultured with a control
25 peptide.

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1 Analysis of decapeptides that supported
2 proliferation of lymphocytes from CFA/I immune animals.
3 Of the 39 different peptides that supported
4 proliferative responses, thirty contained a serine
5 residue, 19 contained a serine at either position 2, 3,
6 or 4, and nine had a serine specifically at position 3.
7 Some of the most robust responses were to the peptides
8 that contained a serine residue at the third position.
9 The amino acid sequence of four such peptides is shown
10 in Table 3.

11 VII. DETAILED DESCRIPTION OF THE INVENTION

12 Applicants have discovered efficacious
13 pharmaceutical compositions wherein the relative
14 amounts of antigen to the polymeric matrix are within
15 the ranges of 0.1 to 1.5% antigen (core loading) and
16 99.9 to 98.5% polymer, respectively. It is preferred
17 that the relative ratio between the lactide and
18 glycolide component of the
19 poly(DL-lactide-co-glycolide) (DL-PLG) is within the
20 range of 40:60 to 0:100. However, it is understood
21 that effective core loads for certain antigens will be
22 influenced by its microscopic form (i.e. bacteria,
23 protozoa, viruses or fungi) and type of infection being
24 prevented. From a biological perspective, the DL-PLG
25 or glycolide monomer excipient are well suited for in
26 vitro drug (antigen) release because they elicit a

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1 minimal inflammatory response, are biologically
2 compatible, and degrades under physiologic conditions
3 to products that are nontoxic and readily metabolized.

4 Surprisingly, applicants have discovered an
5 extremely effective method for the protection against
6 bacterial or viral infections in the tissue of a mammal
7 (human or nonhuman animal) caused by enteropathogenic
8 organisms comprising administering orally to said
9 animal an immunogenic amount of a pharmaceutical
10 composition consisting essentially of an antigen
11 encapsulated within a biodegradable polymeric matrix.
12 When the polymeric matrix is DL-PLG, the most preferred
13 relative ratio between the lactide and glycolide
14 component is within the range of 48:52 to 58:42. The
15 bacterial infection can be caused by bacteria
16 (including any derivative thereof) which include
17 Salmonella typhi, Shigella sonnei, Shigella flexneri,
18 Shigella dysenteriae, Shigella boydii, Escheria coli,
19 Vibro cholera, versinia, staphylococcus, clostridium
20 and campylobacter. Representative viruses contemplated
21 within the scope of this invention, susceptible to
22 treatment with the above-described pharmaceutical
23 compositions, are quite extensive. For purposes of
24 illustration, a partial listing of these viruses
25 (including any derivative thereof) include hepatitis A,
26 hepatitus B, rotaviruses, polio virus human

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1 immunodeficiency viruses (HIV), Herpes Simplex virus
2 type 1 (cold sores), Herpes Simplex virus type 2
3 (Herpesvirus genitalis), Varicella-zoster virus
4 (chicken pox, shingles), Epstein-Barr virus (infectious
5 mononucleosis; glandular fever; and Burkittis
6 lymphoma), and cytomegalo viruses.

7 A further representation description of the
8 instant invention is as follows:

9 A. (1) To homogeneously disperse antigens of
10 enteropathic organisms within the polymeric matrix of
11 biocompatible and biodegradable microspheres, 1
12 nanogram (ng) to 12 microns in diameter, utilizing
13 equal molar parts of polymerized lactide and glycolide
14 (50:50 DL-PLG, i.e. 48:52 to 58:42 DL-PLG) such that
15 the core load is within the range of about 0.1 to 1.5%
16 by volume. The microspheres containing the dispersed
17 antigen can then be used to immunize the intestine to
18 produce a humoral immune response composed of secretory
19 antibody, serum antibody and a cellular immune response
20 consisting of specific T-cells and B-cells. The immune
21 response is directed against the dispersed antigen and
22 will give protective immunity against the pathogenic
23 organism from which the antigen was derived.

24 (2) AF/R1 pilus protein is an adherence
25 factor that allows E. coli RDEC-1 to attach to rabbit
26 intestinal brush borders thus promoting colonization

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1 resulting in diarrhea. AF/R1 pilus protein was
2 homogeneously dispersed within a polymeric matrix of
3 biocompatible and biodegradable microspheres, 1-12
4 microns in diameter (Figure 1 and photograph 1) using
5 equal molar parts of polymerized lactide and glycolide
6 (50:50 DL-PLG) such that the core load was .62% by
7 weight.

8 (3) The microspheres were found to contain
9 immunogenic AF/R1 by immunizing both rabbit spleen
10 (Figure 2) and Peyer's patch (Figure 3) B-cells in
11 vitro. The resultant cell supernatants contained
12 specific IgM antibody which recognized the AF/R1. The
13 antibody response was comparable to immunizing with
14 AF/R1 alone.

15 (4) Microspheres containing 50
16 micrograms of AF/R1 were used to intrainstestinally
17 (intraduodenally) immunize rabbits on two separate
18 occasions 1 week apart. One week later, compared to
19 rabbits receiving AF/R1 alone, the intestinal lymphoid
20 tissue, Peyer's patches, demonstrated an enhanced
21 cellular immune response to AF/R1 and to three AF/R1
22 linear peptide fragments 40-55, 79-94 and 108-123 by
23 both lymphocyte transformation (T-cells) (Figures 4 and
24 5) and antibody producing B-cells (Figures 6 and 7).
25 Similarly enhanced B-cell responses were also detected
26 in the spleen (Figures 8 and 9). An enhanced T-cell

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1 response was also detected in the mesenteric lymph node
2 and the spleen to one AF/R1 peptide fragment, 40-55
3 (Figures 10 and 11). The cellular immune response at
4 two weeks was too early for either a serum or secretory
5 antibody response (See Results in Table 1); but
6 indicates that a secretory antibody response will
7 develop such that the rabbits so immunized could be
8 protected upon challenge with the E. coli RDEC-1.

9 B. Microspheres do not have to be made up just
10 prior to use as with liposomes. Also liposomes have
11 not been effective in rabbits for intestinal
12 immunization of lipopolysaccharide antigens.

13 C. (1) Only a small amount of antigen is
14 required (ugs) when dispersed within microspheres
15 compared to larger amounts (mgms) when antigen is used
16 alone for intestinal immunization.

17 (2) Antigen dispersed within
18 microspheres can be used orally for intestinal
19 immunization whereas antigen alone used orally even
20 with gastric acid neutralization requires a large
21 amount of antigen and may not be effective for
22 intestinal immunization.

23 (3) Synthetic peptides with and without
24 attached synthetic adjuvants representing peptide
25 fragments of protein antigens can also be dispersed
26 within microspheres for oral-intestinal immunization.

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1 Free peptides would be destroyed by digestive processes
2 at the level of the stomach and intestine. Any
3 surviving peptide would probably not be taken up by the
4 intestine and therefore be ineffective for intestinal
5 immunization.

6 (4) Microspheres containing antigen maybe
7 placed into gelatin-like capsules for oral
8 administration and intestinal release for improved
9 intestinal immunization.

10 (5) Microspheres promote antigen uptake
11 from the intestine and the development of cellular
12 immune (T-cell and B-Cell) responses to antigen
13 components such as linear peptide fragments of protein
14 antigens.

15 (6) The development of intestinal T-cell
16 responses to antigens dispersed within microspheres
17 indicate that T-cell immunological memory will be
18 established leading to long-lived intestinal immunity.
19 This long-lived intestinal immunity (T-cell) is very
20 difficult to establish by previous means of intestinal
21 immunization. Failure to establish long-lived
22 intestinal immunity is a fundamental difficulty for
23 intestinal immunization with non-viable antigens.
24 Without intestinal long-lived immunity only a short
25 lived secretory antibody response is established

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1 lasting a few weeks after which no significant
2 immunological protection may remain.

3 D. (1) Oral intestinal immunization of
4 rabbits against E. coli RDEC-1 infection using either
5 whole killed organisms, pilus protein preparations or
6 lipopolysaccharide preparations.

7 (2) Microspheres containing adherence pilus
8 protein AF/R1 or its antigen peptides for oral
9 intestinal immunization of rabbits against RDEC-1
10 infection.

11 (3) Oral-intestinal immunization of humans
12 against enterotoxigenic E. coli infection using either
13 whole killed organisms, pilus protein preparations or
14 lipopolysaccharide preparations.

15 (4) Microspheres containing adherence pilus
16 proteins CFA/I, II, III and IV or their antigen
17 peptides for oral intestinal immunization of humans
18 against human enterotoxigenic E. coli infections.

19 (5) Oral-intestinal immunization of humans
20 against other enteric pathogens as salmonella,
21 shigella, camphlobacter, hepatitis-A virus, rota virus
22 and polio virus.

23 (6) Oral-intestinal immunization of animals
24 and humans for mucosal immunological protection at
25 distal mucosal sites as the bronchial tree in lungs,
26 genito-urinary tract and breast tissue.

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1 E. (1) The biocompatible, biodegradable
2 co-polymer has a long history of being safe for use in
3 humans since it is the same one used in resorbable
4 suture material.

5 (2) By using the microspheres, we are now
6 able to immunize the intestine of animals and man with
7 antigens not normally immunogenic for the intestinal
8 mucosa because they are either destroyed in the
9 intestine, unable to be taken up by the intestinal
10 mucosa or only weakly immunogenic if taken up.

11 (3) Establishing long-lived immunological
12 memory in the intestine is now possible because T-cells
13 are immunized using microspheres.

14 (4) Antigens that can be dispersed into
15 microspheres for intestinal immunization include the
16 following: proteins, glycoproteins, synthetic
17 peptides, carbohydrates, synthetic polysaccharides,
18 lipids, glycolipids, lipopolysaccharides (LPS),
19 synthetic lipopolysaccharides and with and without
20 attached adjuvants such as synthetic muramyl dipeptide
21 derivatives.

22 (5) The subsequent immune response can be
23 directed to either systemic (spleen and serum antibody)
24 or local (intestine, Peyer's patch) by the size of the
25 microspheres used for the intestinal immunization.
26 Microspheres 5-10 microns in diameter remain within

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1 macrophage cells at the level of the Peyer's patch in
2 the intestine and lead to a local intestinal immune
3 response. Microspheres 1 ng - 5 microns in diameter
4 leave the Peyer's patch contained within macrophages
5 and migrate to the mesenteric lymph node and to the
6 spleen resulting in a systemic (serum antibody) immune
7 response.

8 (6) Local or systemic antibody mediated
9 adverse reactions because of preexisting antibody
10 especially cytophyllic or IgE antibody may be minimized
11 or eliminated by using microspheres because of their
12 being phagocytized by macrophages and the antigen is
13 only available as being attached to the cell surface
14 and not free. Only the free antigen could become
15 attached to specific IgE antibody bound to the surface
16 of mast cells resulting in mast cell release of
17 bioactive amines necessary for either local or systemic
18 anaphylaxis.

19 (7) Immunization with microspheres
20 containing antigen leads to primarily IgA and IgG
21 antibody responses rather than an IgE antibody
22 response, thus preventing subsequent adverse IgE
23 antibody reactions upon reexposure to the antigen.

24 In addition to the above, the encapsulation of
25 the following synthetic peptides are contemplated and

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1 considered to be well within the scope of this
2 invention:

- 3 (1) AF/R1 40-55;
4 (2) AF/R1 79-94;
5 (3) AF/R1 108-123;
6 (4) AF/R1 1-13;
7 (5) AF/R1 pepscan 16AA;
8 (6) CFA/I 1-13; and
9 (7) CFA/I pepscan 16AA.
10 (8) Synthetic Peptides Containing CFA/I Pilus

11 Protein

12 T-cell Epitopes (Starting Sequence #
13 given)

14

15 4(Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro),

16

17 8(Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu),

18

19 12(Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

20

21 15(Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala),

22

23 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

24

25 26(Pro-Ser-Ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro),

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1
2 72 (Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser),
3
4 78 (Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln),
5
6 87 (Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe),
7
8 126 (Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr), and
9
10 133 (Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val), and
11 mixtures thereof.

12 (9) Synthetic Peptides Containing CFA/I Pilus
13 Protein B-cell (antibody) Eptiopes (Starting Sequence #
14 given)

15 3 (Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),

16 11 (Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
17 22 (Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

18 32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-
19 Glu-Ser-Tyr-Arg-Val),
20 32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),
21 38 (Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),
22 66 (Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),
23 93 (Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),
24 124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),

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1 127 (Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser),
2 and
3 124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-
4 Ser), and mixtures thereof.
5 (10) synthetic peptides containing CFA/I
6 pilus protein T-cell and B-cell (antibody) epitopes
7 (Starting Sequence # given)
8 3 (Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),
9 8 (Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-A
10 sp),
11 11 (Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
12 20 (Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),
13 124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser
14), and
15 126 (Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and
16 mixtures thereof.
17 (11) synthetic peptides containing CFA/I pilus
18 protein T-cell and B-cell (antibody) epitopes (Starting
19 Sequence # given)

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1 CFA/I pilus protein T-cell epitopes

2

3 4 (Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro) ,

4

5 8 (Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu) ,

6

7 12 (Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp) ,

8

9 15 (Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala) ,

10

11 20 (Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val) ,

12

13 26 (Pro-Ser-Ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro) ,

14

15 72 (Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser) ,

16

17 78 (Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln) ,

18

19 87 (Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe) ,

20

21 126 (Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr) , and

22

23 133 (Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val) ; and

24 synthetic peptides containing CFA/I pilus protein

25 B-cell (antibody) epitopes (Starting Sequence # given)

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1 CFA/I pilus protein B-cell epitopes

2 3 (Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),

3 11 (Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

4 22(Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

5 32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-

6 Glu-Ser-Tyr-Arg-Val),

7 32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),

8 38 (Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),

9 66 (Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),

10 93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),

11 124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),

12 127 (Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser),

13 and

14 124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-

15 Ser), and mixtures thereof.

```

16         (12) synthetic peptides containing CFA/I pilus
17         protein T-cell and B-cell (antibody) epitopes (Starting
18         Sequence # given)

```

19 CFA/I pilus protein T-cell epitopes

20 3 (Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),

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1 11 (Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
2 22 (Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

3 32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-
4 Glu-Ser-Tyr-Arg-Val),
5 32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),
6 38 (Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),
7 66 (Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),
8 93 (Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),
9 124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),
10 127 (Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser),
11 and

12 124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-
13 Ser); and
14 synthetic peptides containing CFA/I pilus protein
15 T-cell and B-cell (antibody) epitopes (Starting
16 Sequence # given)

17 CFA/I pilus protein B-cell epitopes

18 3 (Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),

19 8 (Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-
20 Ala-Asp),

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1 11(Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
2 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

3 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser
4), and

5 126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and
6 mixtures thereof.

7 We contemplate that the peptides can be used in
8 vaccine constructed for systemic administration.

9 VIII. EXAMPLES

10 The peptides in (8), (9), and (10) above can be
11 made by classical solution phase synthesis, solid phase
12 synthesis or recombinant DNA technology. These
13 peptides can be incorporated in an oral vaccine to
14 prevent infection by CFA/I bearing enteropathogenic E.
15 coli.

16 The herein offered examples provide methods for
17 illustrating, without any implied limitation, the
18 practice of this invention in the prevention of
19 diseases caused by enteropathogenic organisms.

20 The profile of the representative experiments
21 have been chosen to illustrate the effectiveness of the
22 immunogenic polymeric matrix-antigen composites.

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1 All temperatures not otherwise indicated are in
2 degrees Celcius (°C) and parts or percentages are given
3 by weight.

4 IX. MATERIALS AND METHODS

5 Animals. New Zealand White male rabbits were
6 purchased from Hazelton Research Products (Denver, PA),
7 and were shown to be free of current RDEC-1 infection
8 by culture of rectal swabs. Animals were 1-2 kg of body
9 weight and lacked agglutinating anti-AF/R1 serum
10 antibody at the time of the study.

11 Antigens. AF/R1 pili from E. coli RDEC-1 (015:H:K
12 non-typable) were purified by an ammonium sulfate
13 precipitation method. The final preparation migrated
14 as a single band on SDS-polyacrylamide gel
15 electrophoresis and was shown to be greater than 95%
16 pure by scanning with laser densitometry when stained
17 with coomassie blue. Briefly, equal molar parts of
18 DL-lactide and glycolide were polymerized and then
19 dissolved to incorporate AF/R1 into spherical
20 particles. The microspheres contained 0.62% protein by
21 weight and ranged in size from 1 to 12 micrometers.
22 Both the microencapsulated and non-encapsulated AF/R1
23 were sterilized by gamma irradiation (0.3 megarads)
24 before use.

25 Synthetic peptides (16 amino acids each) were
26 selected by theoretical criteria from the amino acid

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1 sequence of AF/R1 as deduced from the nucleotide
2 sequence. Three sets of software were used for the
3 selections. Software designed to predict B cell
4 epitopes based on hydrophilicity, flexibility, and
5 other criteria was developed by the University of
6 Wisconsin Genetics Computer Group. Software designed
7 to predict T cell epitopes was based on the Rothbard
8 method was written by Stephen Van Albert (The Walter
9 Reed Army Institute of Research, Washington, D.C.).
10 Software designed to predict T cell epitopes based on
11 the Berzofsky method is published as the AMPHI program.
12 The selected peptides were synthesized by using
13 conventional Merrifield solid phase technology. AF/R1
14 40-55 (Thr-Asn-Ala-Cly-Thr-Asp-Ile-Gly-
15 Ala-Asn-Lys-Ser-Phe-Thr-Leu-Lys) was chosen as a
16 probable B cell epitope for two reasons: (a) due to its
17 high hydrophilic and flexibility indices, and (b)
18 because it was not predicted to be a T cell epitope by
19 either the Rothbard or Berzofsky method. AF/R1 79-94
20 (Val-Asn-Gly-Ile-Gly-Asn-Leu-Ser-Gly-Lys-Ala-Ile-Asp-Al
21 a- His-Val) was selected as a probable T cell eptiope
22 because it contained areas predicted as a T cell
23 epitope by both methods and because of its relatively
24 low hydrophilic and flexibility indices. AF/R1 108-123
25 (Asp-Thr-Asn-Ala-Asp-Lys-
26 Glu-Ile-Lys-Ala-Gly-Gin-Asn-Thr-Val-Asp) was selected

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1 as both a T and B cell epitope. AF/R1 40/47/79-86 was
2 produced in continuous synthesis
3 (Thr-Asn-Ala-Cly-Thr-Asp-Ile-Gly-Val-Asn-GlyIle-Gly-Asn
4 -Leu-Ser) and represents a hybrid of the first eight
5 amino acids from the predicted B cell epitope and the T
6 cell epitope. The purity of each peptide was confirmed
7 by C-8 reverse phase HPLC, and all peptides were
8 desalted over a Sephadex G-10 Column before use. Using
9 a standard ELISA method, all peptides were assayed for
10 their ability to specifically bind anti-AF/R1 IgG
11 antibody in hyperimmune serum from a rabbit which had
12 received intramuscular injections of AF/R1 pili in
13 Freund adjuvant. Only the peptide chosen as a probable
14 B cell epitope (AF/R1 40-55) was recognized by the
15 hyperimmune serum.

16 EXAMPLE 1

17 Immunization. Rabbits were primed twice with 50
18 micrograms of either microencapsulated or
19 non-encapsulated AF/R1 by endoscopic intraduodenal
20 inoculation seven days apart by the following
21 technique. All animals were fasted overnight and
22 sedated with an intramuscular injection of xylazine (10
23 mg) and Ketamine HCl (50 mg). An Olympus BF type P10
24 endoscope was advanced under direct visualization
25 through the esophagus, stomach, and pylorus, and a 2 mm
26 ERCP catheter was inserted through the biopsy channel

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1 and threaded 2-3 cm into the small intestine.
2 Inoculums of pili or pili embedded in microspheres were
3 injected through the catheter into the duodenum and the
4 endoscope was withdrawn. Animals were monitored daily
5 for signs of clinical illness, weight gain, or
6 colonization by RDEC-1.

7 EXAMPLE 2

8 Lymphocyte Proliferation. Seven days following
9 the second priming, the rabbits were again sedated with
10 a mixture of xylazine and katamine HCl, and blood was
11 drawn for serum preparation by cardiac puncture.
12 Animals were then euthanized with an overdose of
13 pentothal and tissues including Peyer's patches from
14 the small bowel, MLN, and spleen were removed. Single
15 cell suspension were prepared and washed in Dulbeco's
16 modified Eagle medium (Gibco Laboratories, Grand
17 Island, NY) which had been supplemented with penicillin
18 (100 units/ml), streptomycin (100 micrograms/ml),
19 L-glutamine (2mM), and HEPES Buffer (10 mM) all
20 obtained from Gibco Laboratories, as well as MEM
21 non-essential amino acid solution (0.1 mM), MEM [50x]
22 amino acids (2%), sodium bicarbonate (0.06%), and 5×10^{-5}
23 micrograms 2-ME all obtained from Sigma Chemical
24 Company (St. Louis, MO) [cDMEM]. Erythrocytes in the
25 spleen cell suspension were lysed using standard
26 procedures in an ammonium chloride lysing buffer. Cell

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1 suspension were adjusted to 5×10^6 cells per ml in
2 cDMEM, and autologous serum was added to yield a final
3 concentration of 0.5%. Cells (0.1 ml) were placed in
4 96-well flat bottom culture plates (Costar, Cambridge,
5 MA) along with 0.1 ml of various dilutions of antigen
6 and were incubated at 37°C in 5% CO₂. In other
7 experiments, cultures were conducted in a 24-well
8 plates. In these experiments, 5×10^6 cells were
9 cultured with or without antigen in a 2 ml volume.
10 After 4 days, 100 microliters aliquots of cells were
11 transferred to 96-well plates for pulsing and
12 harvesting. Previous experiments have demonstrated that
13 optimal concentrations of antigen range from 150 ng/ml
14 to 15 micrograms/ml in the 96-well plate assay and 1.5
15 ng/ml to 150 ng/ml in the 24-well plate assay. These
16 were the concentrations employed in the current study.
17 All cultures were pulsed with 1 Ci [³H]thymidine (25
18 Ci/mmol, Amersham, Arlington Heights, IL) on day 4 of
19 culture and were harvested for scintillation counting 6
20 hours later.

21 Statistics. All cultures were conducted in
22 replicates of four, and standard deviations of the
23 counts per minute (cpm) generally range from 5-15% of
24 the average cpm. In experiments where comparison of
25 individual animals and groups of animals is desirable,
26 data is shown as a stimulation index (SI) to facilitate

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1 the comparison. SI were calculated by dividing the mean
2 of cultures with antigen by the mean of cultures
3 without antigen (media control). Statistical
4 significance (p value) was determined by comparing the
5 maximum response for each antigen to the media control
6 using the Student's t test.

7 IX. RESULTS

8 Lymphocyte proliferation in response to protein
9 and peptide antigens of AF/R1. To determine if
10 lymphoid tissues from AF/R1 immune animals respond in
11 vitro to the antigens of AF/R1, the immunity in a
12 rabbit with preexisting high levels of anti-AF/R1 serum
13 IgG was boosted twice by injection of 50 micrograms of
14 purified AF/R1 pili i.p. seven days apart. A week
15 after the final boost, in vitro lymphocyte
16 proliferation of spleen and MLN cells demonstrated a
17 remarkable response to AF/R1 pili (Fig. 13). In
18 response to the synthetic peptides, there was a small,
19 but significant proliferation of the spleen cells to
20 all the AF/R1 peptides tested as compared to cell
21 cultures without antigen (Fig. 14). Cells from the
22 spleen and Peyer's patches of non-immune animals failed
23 to respond to either AF/R1 or the synthetic peptides.

24 Microencapsulation of AF/R1 potentiates the
25 mucosal cellular immune response. To evaluate the
26 effect that microencapsulation of AF/R1 may have on the

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1 cellular mucosal immune response to that antigen, naive
2 rabbits were primed twice with 50 micrograms of either
3 microencapsulated or non-encapsulated AF/R1 by
4 endoscopic intraduodenal inoculation seven days apart.
5 All rabbits were monitored daily and showed no evidence
6 of clinical illness or colonization by RDEC-1. One
7 week following the last priming, the rabbits were
8 sacrificed and lymphoid tissues were cultured in the
9 presence of AF/R1 pili or peptide antigens. In rabbits
10 which had received non-encapsulated AF/R1, Peyer's
11 Patch cells demonstrated a low level but significant
12 proliferation in vitro in response to AF/R1 pili (Fig.
13 5), but not to any of the AF/R1 synthetic peptides
14 (Fig. 6a-6d). However, in rabbits which had received
15 microencapsulated AF/R1, Peyer's Patch cells
16 demonstrated a markedly enhanced response not only to
17 AF/R1 (Fig. 5), but now responded to the AF/R1
18 synthetic peptides 40-55 and 79-94 (Fig. 6a and 6b).
19 In addition, one of two rabbits primed with
20 microencapsulated AF/R1 (rabbit 135) responded to AF/R1
21 108-123, but not AF/R1 40-47/79-86 (Fig. 6c and 6d).
22 In contrast, the other rabbit in the group (rabbit 134)
23 responded to AF/R1 40-47/79-86, but not to AF/R1
24 108-123 (Fig. 6d and 6c).

25 Response of MLN cells to the antigens of AF/R1.
26 Studies have shown that cells undergoing blastogenesis

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1 in the MLN also tend to home into mucosal areas, but
2 experiments requiring in vitro lymphocyte proliferation
3 of rabbit MLN cells are difficult to conduct and to
4 interpret due to non-specific high background cpm in
5 the media controls. Our studies have shown that this
6 problem can be avoided by conducting the proliferative
7 studies in 24-well plates, and then moving aliquots of
8 cells into 96-well plates for pulsing with
9 [³H]thymidine as described in materials and methods.
10 This method of culture was employed for the remainder
11 of the studies. The MLN cells of all rabbits
12 demonstrated a significant proliferation in vitro in
13 response to AF/R1 pili regardless of whether they had
14 been immunized with microencapsulated or
15 non-encapsulated AF/R1 (Fig. 15). However, only the
16 rabbits which had received microencapsulated AF/R1 were
17 able to respond to the AF/R1 synthetic peptide 40-55
18 (Fig. 11). The MLN cells of rabbit 134 also responded
19 to AF/R1 79-94 ($p < 0.0001$), AF/R1 108-123 ($p < 0.0001$),
20 and AF/R1 40-47/79-86 ($p = 0.0004$); however, none of the
21 other rabbits demonstrated a MLN response to those
22 three peptides (data not shown).

23 Response of spleen cells to the antigens of
24 AF/R1. Proliferative responses of spleen cells to
25 AF/R1 were very weak in all animals tested (data not
26 shown). However, in results which paralleled the

-64-

1 responses in MLN cells, there was a significant
2 response to AF/R1 40-55 in rabbits which had been
3 primed with microencapsulated AF/R1 (Fig. 12). There
4 was no response to the other AF/R1 synthetic peptides
5 by spleen cells in either group of animals. The weak
6 response of spleen cells to AF/R1 provides further
7 evidence that these animals were naive to AF/R1 before
8 the study began, and indicates that the observed
9 responses were not due to non-specific stimulative
10 factors such as lipopolysaccharide.

11 XI. SUMMARY

12 We have shown that there is an enhanced in vitro
13 proliferative response to both protein and its peptide
14 antigens by rabbit Peyer's patch cells following
15 intraduodenal inoculation of antigen which had been
16 homogeneously dispersed into the polymeric matrix of
17 biodegradable, biocompatible microspheres. The
18 immunopotentiating effect of encapsulating purified
19 AF/R1 pili as a mucosal delivery system may be
20 explained by one or more of the following mechanisms:
21 (a) Microencapsulation may help to protect the antigen
22 from degradation by digestive enzymes in the intestinal
23 lumen. (b) Microencapsulation has been found to
24 effectively enhance the delivery of a high
25 concentration of antigen specifically into the Peyer's
26 patch. (c) Once inside the Peyer's patch,

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1 microencapsulation appears to facilitate the rapid
2 phagocytosis of the antigen by macrophages, and the
3 microspheres which are 5-10 micrometers become
4 localized within the Peyer's patch. (d)
5 Microencapsulation of the antigen may improve the
6 efficiency of antigen presentation by decreasing the
7 amount of enzymatic degradation that takes place inside
8 the macrophage before the epitopes are protected by
9 combining with Class II major histocompatibility
10 complex (MHC) molecules. (e) The slow,
11 controlled-release of antigen may produce a depot
12 effect that mimics the retention of antigen by the
13 follicular dendritic cell. (f) If the antigen of
14 interest is soluble, microencapsulation changes the
15 antigen into a particulate form which appears to assist
16 in producing an IgA B cell response by shifting the
17 cellular immune response towards the T_H and thereby not
18 encouraging a response by the T_s . There is evidence
19 that the GALT may be able to discriminate between
20 microbial and non-microbial (food) antigens in part by
21 the form of the antigen when it is first encountered,
22 and thus bacterial antigens do not necessarily have
23 special antigenic characteristics that make them
24 different from food antigens, but they are antigenic
25 because of the bacterial context in which they are
26 presented. The particulate nature of microspheres may

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1 serve to mimic that context. It may be important to
2 note that we also observed a significant response to
3 AF/R1 in animals inoculated with non-encapsulated pili;
4 thus, some of this antigen which was still in its
5 native form was able to enter the Peyer's patch. This
6 may be explained by the fact that AF/R1 is known to
7 mediate the attachment of RDEC-1 to the Peyer's patch
8 M-cell. If the antigen employed in this type of study
9 was not able to attach to micrometer M-cells, one would
10 expect to see an even greater difference in the
11 responses of animals which had received
12 microencapsulated versus non-encapsulated antigen.

13 The microspheres used in these experiments
14 included a size range from 1 to 12 micrometers. The 1
15 to 5 micrometer particles have been shown to
16 disseminate to the MLN and spleen within migrating
17 macrophages; thus, the observed proliferative responses
18 by cells from the MLN and spleen may reflect priming of
19 MLN or splenic lymphocytes by
20 antigen-presenting/accessory cells which have
21 phagocytosed 1 to 5 micrometer antigen-laden
22 microspheres in the Peyer's patch and then disseminated
23 onto the MLN. Alternatively, these responses may be a
24 result of the normal migration of antigen stimulated
25 lymphocytes that occurs from the Peyer's patch to the
26 MLN and on into the general circulation before homing

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1 to mucosal sites. Proliferative responses by MLN cells
2 are of interest because it has been shown that cells
3 undergoing blastogenesis in the MLN tend to migrate
4 onto mucosal areas. However, studies involving in
5 vitro lymphocyte proliferation of rabbit MLN cells can
6 be very difficult to conduct and to interpret due to
7 non-specific high background cpm in the media controls.
8 By simultaneously conducting experiments using
9 different protocols, we have found that this problem
10 can be prevented by avoiding the use of fetal calf
11 serum in the culture and by initially plating the cells
12 in 24-well plates. Using this method, the blasting
13 lymphocytes are easily transferred to a 96-well plate
14 where they receive the [³H]thymidine, while fibroblasts
15 and other adherent cells remain behind and thus do not
16 inflate the background cpm.

17 The proliferative response to the peptide
18 antigens was of particular interest in these studies.
19 The rabbits that received non-encapsulated AF/R1 failed
20 to respond to any of the peptides tested either at the
21 level of the Peyer's patch, the MLN, or the spleen. In
22 contrast, Peyer's patch cells from the animals that
23 received microencapsulated AF/R1 responded to all the
24 peptides tested with two exceptions: Rabbit 134 did
25 not respond to AF/R1 108-123, and rabbit 135 did not
26 respond to AF/R1 40-47/79-86. The reason for these

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1 non-responses is not clear, but it probably is not due
2 to MHC restrictions as evidenced by the fact that
3 rabbit 134 was able to respond to AF/R1 108-123 at the
4 level of the MLN. The non-responses may be due to
5 varying kinetics of sensitized T cell migration in
6 different rabbits, or they may reflect differences in
7 the efficiency of antigen presentation by cells from
8 different lymphoid tissues of these animals. Of all
9 the synthetic peptides tested, only AF/R1 40-55, (the
10 one selected as a probable B cell epitope), was
11 recognized by serum from an AF/R1 hyperimmune rabbit.
12 In addition, this peptide was the only one that was
13 uniformly recognized by Peyer's patch, MLN, and spleen
14 cells from both rabbit. In addition, this peptide was
15 the only one that was uniformly recognized by Peyer's
16 patch, MLN, and spleen cells from both rabbits that
17 were immunized with microencapsulated AF/R1. The
18 recognition by anti-AF/R1 serum antibodies indicates
19 that the amino acid sequence of this peptide includes
20 an immunodominant B cell epitope. Thus AF/R1 40-55 may
21 readily bind to antigen-specific B cells thereby
22 leading to an efficient B cell presentation of this
23 antigen to sensitized T cells. Even though AF/R1 40-55
24 was not selected as a probable T cell epitope by either
25 the Rothbard or Berzofsky methods, the current study
26 clearly indicates that this peptide can also stimulate

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1 a proliferative immune response. Although further
2 studies are required to definitively show that the
3 proliferating cells are indeed T cells, the responses
4 observed in this study are most likely due to the blast
5 transformation of cells from the lineage. Therefore,
6 AF/R1 40-55 appears to contain a T cell epitope in
7 addition to the immunodominant B cell epitope, and this
8 area of the AF/R1 protein may thereby play an important
9 role in the overall immune response and subsequent
10 protection against RDEC-1.

11 The proliferative responses of spleen cells was
12 low in all animals tested; however, we feel tht this
13 may be simply a matter of the kinetics of cellular
14 migration. The rabbits in this study were sacrificed
15 only two weeks after their first exposure to antigen.
16 This relatively short time period may not have provided
17 sufficient time for cells that were produced by Peyer's
18 patch and MLN blasts to have migrated as far as the
19 spleen in sufficient numbers.

20 An ideal mucosal vaccine preparation would not
21 only assist in the uptake and presentation of the
22 immunogen of interst, but it would also be effective
23 without requiring carrier molecules or adjuvants which
24 may complicate vaccine production or delay regulatory
25 approval. The incorporation of antigen into
26 microspheres appears to provide an ideal mucosal

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1 delivery system for oral vaccine immunogens because the
2 observed immunopotentiating effect is achieved without
3 the need for carriers of adjuvants. This ability may
4 prove to be of great value, particularly to enhance the
5 delivery of oral synthetic peptide vaccines to the
6 GALT.

7 TABLE 1. Linear B-Cell Epitopes of CFA/I in Monkeys

8	Sequence	Individuals	
9	<u>Position</u>	<u>Responding</u>	<u>Consensus Site</u>
10	1. 11-21	3	VDPVIDLLQ
11	2. 93-101	2	AKEFEAAA
12	3. 124-136	2	GPAPT
13	4. 66-74	2	PQLTDVLN
14	5. 22-29	2	GNALPSAV
15	6. 32-40	1	KTF*
16	7. 38-45	1	
17	8. 3-11	1	

18

19

*Overlap between epitope 6 and 7

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TABLE 2

1			
2		<u>Prediction of T cell epitopes within the CFA/I</u>	
3		<u>molecule'</u>	
4		<u>Predicted Amphipathic Segments</u>	<u>Rothbard Criteria</u>
5		7 aa blocks	11 aa blocks
6			
7		22-25	8-11
8	16		
9		34-38	32-44
10	30		
11		40-46	51-71
12	38		
13		50-53	86-92
14	44		
15		56-62	102-108
16	57		
17		64-71	130-131
18	61		
19		104-108	135-137
20	70		
21		131-137	
22	116		
23			124
24			127
25			137

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1 *The sequence numbers of the first amino acid of
 2 the predicted T cell epitopes are shown. Software
 3 designed to predict T cell epitopes based on the
 4 Berzofsky method was published as the AMPHI program.
 5 It predicts amphipathic amino acid segments by
 6 evaluating 7 or 11 residues as a block and assigning a
 7 score to the middle residue of that block. Software
 8 designed to predict T cell epitopes based on the
 9 Rothbard method was written by Stephen Van Albert (The
 10 Walter Reed Army Institute of Research, Washington,
 11 D.C.).

TABLE 3

13 Amino acid sequence of immunodominant T cell epitopes*

14	Residue
15	
16	Numbers Amino Acids
17	
18	8-17 Thr Ala Ser Val Asp Pro Val Ile Asp Leu
19	40-49 Phe Glu Ser Tyr Arg Val Met Thr Gln Val
20	72-81 Leu Asn Ser Thr Val Gln Met Pro Ile Ser
21	134-143 Asn Tyr Ser Gly Val Val Ser Leu Val Met

22

23 *Of the 19 decapeptides that supported a
 24 significant proliferative response and contained a
 25 serine at either position 2, 3, or 4, nine has a serine
 26 specifically at position 3. Some of the most robust

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1 responses were to the peptides that contained a serine
2 residue at the third position. The amino acid sequence
3 of four such decapeptides which are believed to be
4 immunodominant T cell epitopes is shown.

5 PHASE III

6 The development of a safe and effective vaccine
7 against enterotoxigenic E. coli (ETEC) would be useful
8 for travelers and for young children in endemic areas.
9 A phase I study of an enteral ETEC vaccine candidate
10 consisting of colonization factor antigen II (CFA/II)
11 encapsulated in biodegradable polymer microspheres
12 (BPM) was conducted in healthy volunteers.

13 Ten adult volunteers swallowed intestinal tubes
14 on days 0, 7, 14, and 28; after collection of jejunal
15 fluid samples, 1 mg of CFA/II in BPM was administered
16 via the tube. Volunteers kept a diary of symptoms
17 after each dose. Secretory IgA in jejunal fluids,
18 serum responses, and antibody secreting cells (ASC)
19 were measured before and after vaccination.

20 The vaccine was well tolerated. Five of 10
21 volunteers had developed IgA anti-CFA/II ASC by 7 days
22 after the last dose of vaccine, these same 5 vaccinees
23 had IgA anti-C63 ASC, and 3 of 5 vaccinees had IgA
24 anti-CS1 ASC. Five of 10 vaccinees developed rises in
25 jejunal fluid sIgA anti-CFA/II with peak CMT of 1:42.
26 Serum responses were meager. Ten vaccinees and 10

-74-

1 unvaccinated control volunteers underwent challenge
2 with 10^9 cfu ETEC E24377A (0139;H2B LT+ST+CS1+CS3+).
3 Ten of 10 controls and 7 of 10 vaccinees developed
4 diarrhea ($p=.11$, 30% vaccine efficacy). One of the 3
5 protected vaccinees had the highest number of ASC and
6 highest sIgA titer before challenge, suggesting that
7 these responses were protective and that this vaccine
8 development strategy has merit. Future studies with
9 higher dosages and a different dosing schedule are
10 planned.

11 Enterotoxigenic Escherichia coli (ETEC) is
12 responsible for diarrhea in infants in developing
13 countries and for a large proportion of diarrhea among
14 travelers to developing countries. Development of a
15 vaccine against ETEC is therefore an important public
16 health priority. Studies in animals and challenged
17 volunteers suggest that orally administered fimbriae,
18 which function as colonization factors, should induce
19 protective immunity.

20 An ETEC vaccine candidate was developed which
21 consists of purified colonization factor antigen II
22 (CFA/II) derived from ETEC strain M424 (06:H16:K15)
23 encapsulated in biodegradable polymer microspheres
24 (BPM). CFA/II from this strain consists of two surface
25 structures, a fibrillar designated coli surface antigen
26 1 (CS1) and a fibrillar structure designated coli

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1 surface antigen 3 (CS3). The purpose of encapsulating
2 the antigen into microspheres is to protect it during
3 passage through the stomach and to enhance its uptake
4 by gut-associated lymphoid tissues (GALT), such as
5 Peyer's patches. The microspheres consist of a 50:50
6 copolymer of lactic and glycolic acids (DL-lactide-co-
7 glycolide). In animals, antigens delivered in these
8 microspheres are taken up and processed by the GALT and
9 stimulate vigorous local immune responses.

10 In this report we describe the safety,
11 Immunogenicity, and efficacy against experimental
12 challenge of the CFA/II-BPM vaccine in healthy
13 volunteers. This is the first use in man of this
14 delivery system for an oral antigen.

15 This phase III describes the result of E. coli
16 CVD 15000, a clinical study of the safety,
17 immunogenicity, and efficacy against experimental
18 challenge of a new vaccine against enterotoxigenic E.
19 coli (ETEC). This vaccine consists of colonization
20 factor antigen II (CFA/II) purified from ETEC strain
21 M424 (06:H16:K15) encapsulated in biodegradable polymer
22 microspheres (CFA/II-BPM).

23 MATERIALS AND METHODS

24 CFA/II-BPM vaccine was prepared at the University
25 of Maryland School of Pharmacy. Each dose of vaccine
26 consisted of 1 mg of CFA/II (90% CS3, 10% CS1)

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1 incorporated into 100 mg of BPM 1. 10 microns in
2 diameter; the freeze-dried microspheres were dispersed
3 in saline containing 0.5% polysorbate₆₀. Ten healthy
4 adult outpatient volunteers were recruited for
5 vaccination with four doses of CFA/II-BPM vaccine.
6 Each volunteer swallowed an intestinal tube on days 0,
7 7, 14, and 28; after collection of jejunal fluid,
8 CFA/II-BPM was administered via the tube. The vaccine
9 vials were sonicated immediately before vaccination to
10 achieve an even suspension of the turbid vaccine.

11 Volunteers kept a diary of symptoms for five days
12 after each dose of vaccine. Jejunal fluids were
13 collected via intestinal tube on days 0, 7, 14, 28, and
14 35 after vaccination for measurement of secretory IgA.
15 Whole blood for antibody secreting cells (ASC) was
16 collected on days 0, 7, 14, 21, and 35. Serum was
17 collected for antibody against CFA/II on days 0, 7, and
18 28. ASC responses were measured by ELISPOT assays
19 using a variety of antigens: CFA/II vaccine antigen
20 derived from ETEC strain M424 (06:H16:K15 CS1+CS3+),
21 purified CS1 derived from ETEC strain 60R75 (O:H CS1+),
22 and purified CS3 derived from ETEC strain E9034 (08:H9
23 CS3+). Four or more spots was considered a significant
24 number. Serum antibody measurements to CFA/II,
25 purified CS1, and purified CS3 were performed by ELISA.
26 A four-fold rise in titer was considered significant.

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1 Jejunal fluids were adjusted to a concentration if IgA
2 of 20 mg% and then lyophilized before assaying for
3 specific anti-CFA/II activity.

4 Fifty-seven days after the first dose of CFA/II-
5 BPM vaccine, 10 vaccinees and 10 unimmunized control
6 volunteers were admitted to the Research isolation Ward
7 in the University of Maryland Hospital. After
8 screening for excellent health, volunteers ingested 3 x
9 10⁹ cfu of ETEC strain E24377A (O139:H28
10 LT+ST+CS1+CS3+) with sodium bicarbonate. Blood samples
11 were collected for serologic responses to CFA/II,
12 O139(LPS) antigen, and heat labile enterotoxin (LT)
13 before and on days 14 and 28 after challenge. Jejunal
14 fluids for measurement of sigA against CFA/II were
15 collected before and on day 7 after challenge.

16 Part I: Outpatient Vaccination Study

17 Ten healthy adult outpatients volunteers were
18 recruited for vaccination with CFA/II-BPM vaccine.
19 Each volunteer swallowed an intestinal tube on
20 September 2, 9, 16, and 30 (days 0, 7, 14, and 28);
21 after collection of jejunal fluid, 1 mg of CFA/II in
22 BPM was administered via the tube. The vaccine was
23 prepared immediately before vaccination as directed by
24 Dr. Reid; specifically, the vials were sonicated to
25 achieve an even suspension of the turbid vaccine. For
26 two volunteers, one or more doses of vaccine had to be

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1 administered intragastrically (noted in data tables)
2 because the tube failed to move out of the stomach
3 after over 56 hours of intubation.

4 **Safety.** Volunteers kept a diary of symptoms for
5 five days after each dose of vaccine. The vaccine was
6 well tolerated. One volunteer reported mild cramps for
7 15 minutes on day 1 after the second dose. A second
8 volunteer reported cramps lasting for about one hour
9 before passing loose stools on days 3 and 4 after the
10 second dose; the volunteer attributed this to having
11 eaten crabs.

12 **Immunogenicity.** Jejunal fluids were collected
13 via intestinal tube on days 0, 8, 14, 28, and 35 after
14 vaccination for measurement of secretory IgA. Whole
15 blood for antibody secreting cells (ASC) was collected
16 on days 0, 7, 14, 21, and 35. Serum was collected for
17 antibody determinations on days 0, 7, and 28. Whole
18 blood for measuring T cell responses by lymphocyte
19 transformation were drawn on days 0 and 35 after
20 vaccination.

21 **ASC.** Detection of CFA/II-specific antibody
22 secreting cells in peripheral blood reflects priming of
23 the intestinal mucosal immune system; these cells have
24 been stimulated by oral antigen, entered the
25 circulation, and are returning to the mucosa to provide

-79-

1 local immunicyt. The role of these cells in protection
2 against ETEC diarrhea is unknown.

3 We measured ASC responses by ELISPOT assays
4 using a variety of antigens: CFA/II vaccine antigen
5 derived from ETEC strain M424 (06:H16:K15 CS1+CS3+),
6 purified CD1 derived from ETEC strain 60R75 (0:H CS1+),
7 purified CS3 derived from ETEC strain E9034 (08:H9
8 CS3+), CS3 peptide 795, CS3 peptide 792, and as
9 controls, CFA/I, CFA/I peptide 791, and CFA/I peptide
10 900. The results of these assays are shown in Tables 1
11 through 5. Four or more spots is considered a
12 significant number.

13 At day 7 after the first dose of vaccine, four of
14 the 10 volunteers developed IgA ASC against CFA/II
15 (Table 1). After the second and third doses of vaccine
16 no additional responders were detected. However, after
17 the fourth dose, an additional volunteer developed a
18 significant response so that the overall response after
19 four doses of CFA/II-BPM was five (50%) of 10
20 vaccinees.

21 Three of the volunteers who responded with IgA
22 ASC against CFA/II also had IgA ASC against purified
23 CS1 (Table 8). The same five volunteers who responded
24 to CFA/II also had IgA ASC against purified CS3 (Table
25 9). This suggests that the responses to CFA/II were
26 specific and not directed against contaminating

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1 elements such as LPS, since the serotypes of the
2 strains from which the antigens were prepared were
3 different. IgA ASC responses to two peptides derived
4 from CS3 were meager or absent (Tables 10 and 11).
5 There were no ASC responses to CFA/I or to two
6 peptides derived from CFA/I. This is further evidence
7 that the responses to CFA/II were not directed against
8 contaminating elements in the antigen preparations.

9 Jejunal fluid sigA. After the first dose of
10 CFA/II-BPM vaccine, only one volunteer developed a rise
11 in sigA to CFA/II and this volunteer (15001-9) had
12 evidence of previous priming since his pre-vaccination
13 sigA anti-CFA/II titer was 1:16 (Table 12). One week
14 after the fourth dose (day 35), however, five of the 10
15 vaccinees had developed rises in sigA anti-CFA/II.
16 Among these five converters, the peak geometric mean
17 titer was 1:42.

18 **Serology.** Serum antibody measurements to
19 CFA/II, purified CS1, and purified CS3 were also
20 performed by ELISA. A four-fold rise in titer was
21 considered significant and indicated by a + in the
22 tables. There was a high prevalence of serum antibody
23 to CFA/II before vaccination (Table 13); only two of 10
24 volunteers developed rises in serum IgA anti-CFA/II and
25 a third volunteer developed a rise in serum IgG anti-
26 CFA/II. Only one volunteer developed serum antibody to

-81-

1 CS1 (Table 14). However, six of the 10 vaccinees
2 developed seroconversions to anti-CS3 with antibody of
3 at least one isotpy (Table 15).

4 **Lymphocyte proliferation studies.** Lymphocytes
5 were separated from whole blood on ficoll-hypaque
6 gradients and stored forzen for future proliferative
7 assays by Dr. Reid at WRAIR.

8 **Part II: Experimental ETEC Challenge Study**

9 All 10 vaccinees and 10 control volunteers agreed
10 to participate in an ETEC challenge. One October 29,
11 1992, 57 days after the first dose of CFA/II-BPM
12 vaccine, 20 volunteers ingested 3×10^9 cfu of ETEC
13 strain E24377A (0139:H28 LT+ST+CS1+CS3+). The clinical
14 and bacteriologic responses to challenge are shown in
15 Table 10.

16 Ten of 10 control volunteers and seven of 10
17 vaccines developed diarrhea ($p=0.11$, Fisher's exact
18 test, 1-tailed; 30% vaccine efficacy). the mean volume
19 of diarrheal stools was 1464 ml for controls and 2819
20 ml for vaccines ($p=0.2$, Student's t test); the mean
21 number of diarrheal stools was 8.6 for controls and
22 14.7 for vaccinees ($p=0.2$, Student's t test). The mean
23 incubation periods in the two groups were not
24 significantly different. The duration of stool
25 shedding and the peak stool excretion of challenge
26 organisms were not significantly different. The three

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1 protected vaccinees had a somewhat lower peak excretion
2 of challenge organisms than the seven unprotected
3 vaccinees, but this difference was not statistically
4 significant.

5 Before challenge (day 57 after the first dose of
6 vaccine), the three protected vaccinees, five vaccinees
7 who became ill, and four control volunteers who became
8 ill had circulating ASC producing antibodies of some
9 isotype against CFA/II, CS1, or CS3 (Table 17). The
10 vaccinee (volunteer 15001-9) with the highest number of
11 IgA anti-CFA/II ASC (240 spots) before challenge and
12 the highest number of IgA anti-CS3 ASC (16 spots)
13 before challenge was one of the three protected
14 vaccinees. The other protected vaccinees (volunteers
15 15001-6 and 15001-11) had no detectable anti-CFA/II IgA
16 ASC before challenge but did have anti-CS1 ASC or anti-
17 CS3 IgA ASC. Conversely, unvaccinated control
18 volunteers with pre-existing IgA anti-CFA/II ASC were
19 not protected (e.g., volunteers 15002-8, 15002-11, and
20 15002-13).

21 The level of ASC response inducted by infection
22 provides a target for future vaccine-induced immunity.
23 After wild-type challenge of vaccinees and controls,
24 IgA ASC responses to CFA/II and CS3 were vigorous
25 (range 12-408 spots for CFA/II and 14-712 spots for
26 CS3) (Table 17). After challenge one vaccinee and one

-83-

1 control volunteer mounted ASC responses to CS3 peptide
2 792 (Table 18). Four vaccinees (15001-1, 15001-6,
3 15001-7, and 15001-11) and one control volunteer
4 (15002-11) developed a small number of ASC to CS3
5 peptide 795 (Table 18).

6 There was no correlation between pre-existing
7 anti-LPS ASC and protection (Table 19). None of the
8 three protected vaccinees had such antibodies before
9 challenge. Two volunteers with pre-existing anti-LPS
10 ASC nevertheless became ill (volunteers 15001-1 and
11 15001-8). Similarly, there was not correlation between
12 protection against illness and pre-existing anti-LT ASC
13 (Table 19).

14 The serologic responses and jejunal fluid
15 antibody responses to challenge are pending at the time
16 of this writing. These results will be summarized in
17 an addendum to this report.

18 RESULTS

19 Clinical and immunologic responses to
20 vaccination. The vaccine was well tolerated. For two
21 volunteers, four doses of vaccine had to be
22 administered intragastrically in two volunteers because
23 the tube failed to move out of the stomach after over
24 56 hours of intubation.

25 Detection of CFA/II-specific antibody secreting
26 cells in peripheral blood reflects priming of the

-84-

1 intestinal mucosal immune system; these cells have been
2 stimulated by oral antigen, entered the circulation,
3 and are refurring to the mucosa to provide local
4 immunity. At day 7 after the first dose of vaccine,
5 four of the 10 volunteers developed IgA ASC against
6 CFA/II. Ater the second and third doses of vaccine no
7 additional responders were detected. However, after
8 the fourth dose, an additional volunteer developed a
9 significant response so that the overall response after
10 four doses of CFA/II-BPM was five (50%) of 10 vaccinees
11 by day 35 (Table 20). Three of the volunteers who
12 responded with IgA ASC against CFA/II also had IgA ASC
13 against purified CS1 (Table 20). The same five
14 volunteers who responded to CFA/II also had IgA ASC
15 against purified CS3 (Table 20). This suggests that
16 the responses to CFA/II were specific and not directed
17 against contaminating elements such as LPS, since the
18 serotypes of the strains from which the antigens were
19 prepared were different.

20 After the first dose of CFA/II-BPM vaccine, only
21 one volunteer developed a rise in jejunal fluid sigA to
22 CFA/II, and this volunteer had evidence of previous
23 priming since his pre-vaccination sigA anti-CFA/II
24 titer was 1:16. One week after the fourth dose (day
25 35), however, five of the 10 vaccinees had developed

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1 rises in sigA anti-CFA/II (Table 20). Among these five
2 converters, the peak geometric mean titer was 1:42.

3 There was a high prevalence of serum antibody to
4 CFA/II before vaccination; only two of 10 volunteers
5 developed rises in serum IgA anti-CFA/II and a third
6 volunteer developed a rise in serum IgG anti-CFA/II.
7 Only one volunteer developed serum antibody to CS1.
8 However, six of the 10 vaccinees developed
9 seroconversions to anti-CS3 with antibody of at least
10 one isotype.

11 **Clinical and bacteriologic responses to**
12 **experimental ETEC challenge.** Fifty-seven days after
13 the first dose of CFA/II-BPM vaccine, 10 vaccinees and
14 10 control volunteers ingested 3×10^9 cfu of ETEC
15 strain E24377A (0139:H28 LT⁺ST⁺CS1⁺CS3⁺). The
16 immunologic status at the time of challenge and the
17 clinical and bacteriologic responses to challenge are
18 shown in Table 22.

19 Ten of 10 control volunteers and seven of 10
20 vaccinees developed diarrhea ($p=0.11$, Fisher's exact
21 test, 1-tailed; 30% vaccine efficacy). The mean volume
22 of diarrheal stools was 1464 ml for controls and 2819
23 ml for vaccines ($p=0.2$, Student's t test); the mean
24 number of diarrheal stools was 8.6 for controls and
25 14.7 for vaccinees ($p=0.2$, Student's t test). The mean
26 incubation periods in the two groups were not

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1 significantly different. The duration of stool
2 shedding and the peak stool excretion of challenge
3 organisms were not significantly different.

4 On the day of challenge, 8 of 10 vaccinees and 4
5 of the 10 control volunteers had circulating IgA ASC
6 producing antibodies against CFA/II, CS1, and/or CD3.
7 The apparent development of additional ASC responders
8 on day 57 after the first dose of vaccine (making the
9 total number of vaccine responders 8 of 10) was
10 unexpected. The high prevalence of anti-colonization
11 factor ASC in control volunteers before challenge was
12 also unexpected and not observed in previous groups of
13 North American volunteers. The vaccinees with the
14 highest number of IgA anti-CFA/II ASC (240 spots), the
15 highest number of IgA anti-CS3 ASC (16 spots), and the
16 highest sigA anti-CFA/II titer (1:256) before challenge
17 was one of the three protected vaccinees. Conversely,
18 the 4 unvaccinated control volunteers with pre-existing
19 IgA anti-CFA/II ASC (range 8-32 spots) were not
20 protected; none of these had pre-existing sigA measured
21 in jejunal fluid before challenge.

22 There was no correlation between pre-existing
23 anti-LPS ASC and protection. Similarly, there was no
24 correlation between protection against illness and pre-
25 existing anti-LT ASC.

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1 Immune responses after wild-type challenge, which
2 are likely to be protective against subsequent
3 challenge, are a target for vaccine-induced immunity.
4 The immune responses in volunteers after 4 doses of
5 CFA/II-BPM vaccine (Table 20) can be compared to those
6 of unimmunized control volunteers after challenge
7 (Table 21). Responses after this vaccine regimen
8 occurred at a lower rate and were of lower magnitude
9 than those achieved after a vigorous wild-type
10 challenge.

11 DISCUSSION

12 CFA/II-BPM vaccine was well tolerated in adult
13 volunteers. When immune responses were measured by the
14 presence of IgA ASC or jejunal fluid sigA, both
15 measured 7 days after the fourth dose, half the
16 volunteers responded to four doses of 1 mg CFA/II-BPM
17 per dose. The vaccine conferred 30% protective
18 efficacy against a rigorous experimental challenge that
19 produced an attach rate of 100% in control volunteers.

20 The three protected vaccinees did not differ
21 significantly from the seven unprotected vaccinees, at
22 least in the immune parameters measured in this study.
23 However, the volunteer who had the highest number of
24 ASC against CFA/II and CS3 and the highest sigA titers
25 among the vaccinees was one of the 3 vaccinees who did

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1 not become ill. This suggests that these immune
2 responses contributed to protection.

3 Some volunteers had a significant number of IgA,
4 IgG, or IgM ASC to CFA/II and/or CS3 on day 57 after
5 the first dose of vaccine (the day of challenge) that
6 were not present on day 35 after vaccination. This
7 suggests that the biodegradable polymer microspheres
8 may have persisted in the submucosa and continued to
9 stimulate responses beyond the 7 to 10 days when ASC
10 responses are ordinarily expected. However, some
11 control volunteers also had ASC responses to CFA/II
12 before challenge. No technical difficulty with the ASC
13 assay could be identified and control blank wells did
14 not react. Confirmation of the presistence of CFA/II-
15 BPM vaccine with continued induction of immune
16 responses will await future studies.

17 The modest efficacy of CFA/II-BPM vaccine may be
18 related to the very small doseage (1 mg of Cfa/II x 4
19 doses) given. The responses after ETEC challenge
20 summarized in Table 21. However, are within reach,
21 perhaps by increasing the dose or changing the schedule
22 of vaccination. Future studies should also include
23 evaluation of the oral route of administration because
24 of the impracticality of delivering vaccine via
25 intestinal tube.

26 DEMONSTRATIVE EVIDENCE OF PROTECTIVE IMMUNITY

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1 RDEC-1 is an eteroadherent diarrhea producing E.
2 coli in rabbit. Its attachment to the mucosa is by the
3 adhesin (AF/R1 pili). The adhesin is an excellent
4 vaccine candidate. It may intitiate a mucosal response
5 but is susceptiple to digestion in the gut. The
6 incorporation of AF/R1 into biocompabile,
7 nondigestible microspheres enhanced mucosal cellular
8 immune respones to RDEC-1. We have demonstrated that
9 immunization with AF/R1 Pili in microspheres protect
10 rabbits against infection with RDEC-1.

11 Six rabbits received intra-duodenal immunizaiton
12 of AF/R1 microspheres (0.62% coreloading by weight) at
13 200 ug AF/R1 on day 0 then boosted with 100 ug AF/R1 in
14 microspheres on days 7, 14, and 21 followed by RDEC-1
15 challenge with 10^8 organisms one week latter than
16 observed for 1 week and then sacrificed, unimmunized
17 rabbits were challenged with 10^8 RDEC-1 only and
18 observed 1 week than sacrificed. Also, 2 rabbits were
19 immunized only then were sacrificed 10 days latter.
20 Only one of these animals had bile IgA antibodies to
21 AF/R1. but both had specific sensitized T cells which
22 released IL-4 upon challenge in the spleen, Peyer's
23 patch and illeal lamina propria. All nine immunized
24 animals developed diarrhea and weight loss which was
25 significant at the $p < .001$ level compared to the
26 immunized animals which displayed no diarrhea and no

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1 weight loss. The immunized animals colonized the
2 intestinal tract with RDEC-1 the same as the
3 unimmunized animals. However, there was a striking
4 difference regarding the adherence of RDEC-1 to the
5 mucosa. No adherence was seen in cecum in the
6 immunized animals compared to 4/7 in the unimmunized
7 side animals. This difference was significant to the p
8 < .01 level. The RDEC-1 exposure although not
9 producing disease in the immunized animals did effect a
10 booster immunization as relected in the increase in
11 anti-AF/R1 antibody containing cells in the muscosa
12 similiar to the immunized rabbits. This study clearly
13 demonstrated complete protection against RDEC-1
14 infection and strongly indicates similiar results
15 should be expected with enterotoxigenicity E. coli using
16 the Colony Forming Antigens (CFA's) in microsphere
17 vaccines.

18 SUMMARY STATEMENT OF PROTECTIVE IMMUNITY SHOWINGS

19 RDEC-1 infection of rabbits causes an
20 enteroadherent E. coli diarrheal disease, and provides
21 a model for the study of adherence-factor immunity.
22 Pilus adhesions are vaccine candidates, but purified
23 pili are subject to intestinal degradation. Previously
24 we showed potentiation of the mucosal cellular immune
25 response to the AF/R1 pilus of RDEC-1 by incorporation
26 into biodegradable polylactide-coglycolide microspheres

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1 (AF/R1-MS). We now present efficacy testing of this
2 vaccine. Six rabbits were primed with 200 ug and
3 boosted with 100 ug of AF/R1-MS weekly x3, then
4 challenged at week 5 with 10^8 CFU of RDEC-1 expressing
5 AF/R1. Nine unvaccinated rabbits were also challenged.
6 Two rabbits vaccinated with AF/R1-MS were sacrificed at
7 week 5, without challenge, for measurement of
8 anti-AF/R1 antibodies in bile (by ELISA) and anti-AF/R1
9 containing cells (ACC) in the intestinal lamina propria
10 (by immunohistochemistry). Attachment of RDEC-1 to
11 intestinal epithelial cells was estimated (0.4+) by
12 immunoperoxidase staining of histologic sections.
13 Colonization of intestinal fluid was measured by
14 culture of intestinal flushes. Results: Rabbits given
15 AF/R1-MS remained well and 4/6 gained weight after
16 challenge, whereas 9/9 unvaccinated rabbits lost weight
17 after challenge (mean weight change +10 vs -270 gms
18 $p < .001$), (see Figure 27). The mean score of RDEC-1
19 attachment to the cecal epithelium was 0 in vaccinated,
20 and 2+ in unvaccinated animals (see Figure 28). RDEC-1
21 colonization (log CFU/gm) in cecal fluids was similar
22 in both groups (mean 6.3 vs 7.3; $p = .09$) (see Figure
23 26). ACC were not seen in the lamina propria of
24 vaccinated but unchallenged animals, but anti-pilus IgA
25 antibody levels in bile were increased 1 S.D. over
26 negative controls in 1 animal. Conclusions:

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1 Vaccination with AF/R1-MS was safe and protected
2 rabbits against RDEC-1 disease. Protection was
3 associated with interference with RDEC-1 adherence to
4 the mucosal surface, but luminal colonization was not
5 prevented.

6 More recently, applicants have focused on areas
7 of this invention related to an immunostimulating
8 composition comprising encapsulating microspheres,
9 which may contain a pharmaceutically-acceptable
10 adjuvant, wherein said microspheres are comprised of
11 (a) a biodegradable-biocompatible poly (DL-lactide-co-
12 glycolide) as the bulk matrix, wherein the relative
13 ratio between the amount of lactide and glycolide
14 components are within the range of 40:60 to 0:100 and
15 (b) an immunogenic substance comprising Colony Factor
16 Antigen (DFA/II, hepatitis B surface antigen (HBsAg),
17 or a physiologically similar antigen that serves to
18 elicit the production of antibodies in mammalian
19 subjects.

20 These areas of invention are referred to herein
21 after as Phase III and Phase IV, respectively, and are
22 summarized as follows:

23 1. An immunostimulating composition comprising
24 encapsulating- microspheres, which may contain a
25 pharmaceutically-acceptable adjuvant, wherein said
26 microspheres having a diameter between 1 nanogram (ng)

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1 to 10 microns (um) are comprised of (a) a
2 biodegradable-biocompatible poly (DL-lactide-co-
3 glycolide) as the bulk matrix, wherein the relative
4 ratio between the amount of lactide and glycolide
5 components are within the range of 40:60 to 0:100 and
6 (b) an immunogenic substance comprising Colony Factor
7 Antigen (CFA/II), hepatitis B surface antigen (HBsAg),
8 or a physiologically similar antigen that serves to
9 elicit the production of antibodies in animal subjects.

10 2. An immunostimulating composition according to
11 Claim 1 wherein the amount of said immunogenic
12 substance is within the range of 0.1 to 1.5% based on
13 the volume of said bulk matrix.

14 3. An immunostimulating composition according to
15 Claim 2 wherein the relative ratio between the lactide
16 and glycolide component is within the range of 48:52 to
17 58:42.

18 4. An immunostimulating composition according to
19 Claim 2 wherein the size of more than 50% of said
20 microspheres is between 5 to 10 um in diameter by
21 volume.

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1 5. An immunostimulating composition according
2 to Claim 1 wherein the immunogenic substance is the
3 synthetic peptide representing the peptide fragment
4 beginning with the amino acid residue 63 through 78 of
5 Pilus Protein CS3, said residue having the amino acid
6 sequence, 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-His-
7 Glu-Thr-Asn-Asn-Ser-Ala).

8 6. A vaccine comprising an immunostimulating
9 composition of Claim 4 and a sterile, pharmaceutically-
10 acceptable carrier therefor.

11 7. A vaccine comprising an immunostimulating
12 composition of Claim 6 wherein said immunogenic
13 substance is Colony Factor Antigen (CFA/II).

14 8. A vaccine comprising an immunostimulating
15 composition of Claim 6 wherein said immunogenic
16 substance is hepatitis B surface antigen (HBsAg).

17 9. A method for the vaccination against
18 bacterial infection comprising administering to a
19 human, an antibactericidally effective amount of a
20 composition of Claim 6.

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1 10. A method according to Claim 8 wherein the
2 bacterial infection is caused by a bacteria selected
3 from the group consisting essentially of Salmonella
4 typhi, Shigella Sonnei, Shigella Flexneri, Shigella
5 dysenteriae, Shigella boydii, Escheria coli, Vibrio
6 cholera, yersinia, staphylococcus, clostridium, and
7 campylobacter.

8 11. A method for the vaccination against viral
9 infection comprising administering to a human an
10 antivirally effective amount of a composition of Claim
11 8.

12 12. A diagnostic assay for bacterial infections
13 comprising a composition of Claim 4.

14 13. A method of preparing an immunotherapeutic
15 agent against infections caused by a bacteria
16 comprising the step of immunizing a plasma donor with a
17 vaccine according to Claim 7 such that a hyperimmune
18 globulin is produced which contains antibodies directed
19 against the bacteria.

20 14. A method preparing an immunotherapeutic
21 agent against infections caused by a virus comprising
22 the step of immunizing a plasma donor with a vaccine

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1 according to Claim 8 such that hyperimmune globulin is
2 produced which contains antibodies directed against the
3 hepatitis B virus.

4 15. An immunotherapy method comprising the step
5 of administering to a subject an immunostimulatory
6 amount of hyperimmune globulin prepared according to
7 Claim 13.

8 16. An immunotherapy method comprising the step
9 of administering to a subject an immunostimulatory
10 amount of hyperimmune globulin prepared according to
11 Claim 14.

12 17. A method for the protection against
13 infection of a subject by enteropathogenic organisms or
14 hepatitis B virus comprising administering to said
15 subject an immunogenic amount of an immunostimulating
16 composition of Claim 3.

17 18. A method according to Claim 17 wherein the
18 immunostimulating composition is administered orally.

19 19. A method according to Claim 17 wherein the
20 immunostimulating composition is administered
21 parenterally.

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1 20. A method according to Claim 17, wherein the
2 immunostimulating composition is administered in four
3 separate doses on day 0, day 7, day 14, and day 28.

4 21. A method according to Claim 17 wherein the
5 immunogenic substance is the synthetic peptide
6 representing the peptide fragment beginning with the
7 amino acid residue 63 through 78 of Pilus Protein CS3
8 said residue having the amino acid sequence 63(Ser-Lys-
9 Asn-Gly-Thr-Val-Thr-Try-Ala-His-Glu-Thr-Asn-Asn-Ser-
10 Ala).

11 PHASE III

In sum, the Colony Factor Antigen (CFA/II) from enterotoxigenic E coli (ETEC) prepared under GMP was successfully incorporated into biodegradable polymer microspheres (CFA/II BPM) under GMP and found to be safe and immunogenic when administered intra-duodenally to rabbits. CFA/II was incorporated into poly (D,L-lactide-co-glycolide) (PLGA) microspheres which were administered by direct endoscopy into the duodenum. Following vaccination, Peyer's patch cells responded by lymphocyte proliferation to in vitro challenge with CFA/II indicating the CFA/II BPM to be immunogenic when administered intra-intestinally. Also, B cells secreting specific anti CFA/II antibodies were found in

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1 spleens following vaccination. No pathological changes
2 were found following total necropsies of 10 rabbits
3 vaccinated with CFA/II BPM. As a potency test, high
4 serum IgG antibody titers to CFA/II were produced
5 following intra- muscular administration of CFA/II BPM
6 to additional rabbits. The CFA/II BPM contained 63%
7 between 5-10 um by volume particle size distribution;
8 1.17% protein content; 2.15% moisture; <.01%
9 acetonitrile; 1.6% heptane; 22 nonpathogenic bacteria
10 and 3 fungi per 1 mgm protein dose; and passed the
11 general safety test. We conclude that the CFA/II BPM
12 oral vaccine is immunogenic and safe to begin a Phase I
13 clinical safety study following IND approval.

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) causes diarrheal disease with an estimated 650,000,000 cases annually in developing countries resulting in 500,000 deaths predominantly in the pediatric age groups. Currently there is no vaccine against ETEC induced diarrhea. The availability of an effective oral vaccine would be of great value to the people of South America, Africa and Asia as well as the millions of people who travel to these high risk areas and account for half of the annual cases.

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1 The first step in pathogenesis is adherence to
2 the small intestine epithelial cells by protein
3 fimbrial (pilus) adhesins called colonization factor
4 antigen (CFA). Three major CFAs have been recognized,
5 CFA/I, CFA/II and CFA/IV. (25)

6 Ten human volunteers who were immunized orally
7 twice weekly for 4 weeks with CFA/II developed a poor
8 antibody response and did not show any significant
9 protection when challenged with pathogenic ETEC (26).
10 This disappointing response was attributed to adverse
11 effects of gastric acid, even at neutral pH, of
12 fimbrial proteins (27). When the vaccine was
13 administered by inoculation directly into the duodenum,
14 4 of 5 immunized volunteers developed a significant
15 rise in secretory IgA with CFA/II antibody (26).

16 D and L-lactic acid and glycolic acid, as homo-
17 and copolymers, are biodegradable and permit slow and
18 continued release of antigen with a resultant adjuvant
19 activity. These polymers have been shown to be safe in
20 a variety of applications in human beings and in
21 animals (28-32). Delivery of antigens via microspheres
22 composed of biodegradable, biocompatible lactide/
23 glycolide polymers (29-32) may enhance the mucosal
24 response by protecting the antigen from digestion and

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1 targeting them to lymphoid cells in Peyer's patches
2 (29-32). McQueen et al. (33) have shown that E coli
3 AF/R1 pili in PLGA microspheres, introduced intra-
4 duodenally in rabbits, protected them against diarrhea
5 and weight loss when challenged with the parent strain
6 rabbit diarrheagenic strain of E coli (RDEC-1). Only
7 one vaccinated rabbit of six lost weight and only one
8 had soft pelleted stool. In contrast, all control
9 unvaccinated animals became ill, lost weight, and shed
10 soft pellets or unformed mucoid stool. Significant
11 lymphocyte proliferation to AF/R1 from Peyer's patches
12 and ordinary IgA anti AF/R1 antibody levels were seen.

13 In order to improve the CFA/II vaccine it was
14 incorporated into PLGA microspheres under GMP in order
15 to protect it from digestion and target it to the
16 intestinal lymphoid system. The CFA/II BPM vaccine has
17 undergone pre-clinical evaluation and has been found to
18 be safe and immunogenic.

19 MATERIALS AND METHODS

20 Preparation of CFA/II Pilus Vaccine. Under Good
21 Laboratory and Good Manufacturing Practices, E. coli.
22 strain M424C1-06;816 producing CFA/II were cultured in
23 75-80 CFA agar plates (24 x 24 cm) for 24 hrs then
24 harvested by scraping. The harvest was homogenized at

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1 slow speed for 30 minutes with over head drive unit and
2 cup immersed in an ice bath. The homogenate was
3 centrifuge at 4° C at 16, 500 x g for 30 minutes. The
4 supernatant saved and the pellet rehomogenized and
5 centrifuged with the supernatants pooled. The
6 supernatant pool was centrifuged at 50,000 x g for 45
7 minutes. The supernatant treated with ammonium sulfate
8 at 20% saturation, stirred 30 minutes at 4° C than
9 stored at 4° C for 16 hrs then centrifuged at 19,700 x
10 g for 30 minutes. The supernatant saved and treated
11 with ammonium sulfate at 45% saturation, stirred 30
12 minutes at 4° C, stored at 4° C for 66-72 hrs, then
13 centrifuged at 19,700 x g for 45 minutes. The pellet
14 was resuspended in about 100 mls of PBS containing 0.5%
15 formalin and held at 22° for 18 hrs then dialyzed for
16 45-50 hrs against PBS at 4° C using a total of 12
17 liters in 2 liter amounts. The dialysis was terminated
18 when the PBS contained less then 0.03% formalin using
19 Nessler's reagent and fuchsin sulfuose acid reagent.
20 The final product contained 1 mgm protein/ml PBS, was
21 sterile and passed the general safety test.

22 Preparation of Desalted CFA/II Vaccine. Two ml
23 of the CFA/II vaccine were placed into a Centricon 30
24 tube and centrifuged at 1700 rpm at 4-6° C (Beckman
25 model GPR centrifuge equipped with GA-24fixed angle
26 rotor) until all the buffer solution passed through the

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1 filter (about 90-120 minutes). Sterile water was added
2 to each tube to disperse the CFA/II retained on the
3 filter. The desalted antigen dispersions from all tube
4 were pooled and then divided into five equal parts by
5 weight so as to contain 20 mg of the CFA/II each. The
6 desalted antigen dispersion was stored at -10 to -20°
7 C.

8 Freeze Drying of the Desalted CFA/II Dispersion.

9 80 mg of sucrose was added to each part of the CFA/II
10 dispersion. The resulting mixture was flash-frozen
11 using a dry ice-acetone bath (100-150 ml of acetone and
12 50-100 g of dry ice). The frozen solution was freeze
13 dried overnight using Repp Sublimator 16 freeze dryer
14 at vacuum of 1 micrometer of mercury and a shelf
15 temperature not exceeding 37° C.

16 CFA/II Biodegradable Polymer Microspheres.

17 Particle size distribution. About 1 mgm of
18 microspheres were dispersed in 2 ml of 1% Polysorbate
19 60° (Ruger Chemical Co. Inc. Irvington, N.J.) in water
20 in a 5 ml capacity glass vial by sonication. This
21 dispersion was observed under a calibrated optical
22 microscope with 43x magnification. Using a
23 precalibrated eye-piece micrometer, the diameter of 150
24 randomly chosen microspheres, was determined and the
25 microsphere size distribution was determined

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1 Scanning Electron Microscopic Analysis.

2 Microspheres were sprinkled on the surface of 10mm stub
3 covered with a non-conductive adhesive (Sticky-Tab,
4 Ernest F. Fullem, Inc., Lutham, N.Y.) Samples were
5 coated with gold/palladium in an automatic sputter-
6 coating apparatus (Samsputter-2A, Tonsimis Research
7 Corporation). The samples were examined with a Hitachi
8 S-450 scanning electron microscope operated at 15-20
9 KV.

10 Preparation of CFA/II Microspheres. Solvent
11 extraction technique was used to encapsulate the freeze
12 dried CFA/II into poly(lactide-co-glycolide) (Medisorb
13 Technologies International, viscosity 0.73 dl/g)
14 microspheres in the 1-10 um size range to achieve
15 theoretical antigen loading of 1% by weight. The
16 freeze dried antigen-sugar & matrix was dispersed in an
17 acetolnitrile solution of the polymer and then
18 emulsified to achieve desired droplet size.
19 Microspheres were solidified and recovered by using
20 heptane as extracting solvent. The microsphere batches
21 were pooled and vacuum dried to remove traces of
22 solvent.

23 Protein Content. The CFA/II microspheres were
24 dissolved in 0.9% SDS in 0.1N NaOH for 18 hr with
25 stirring then neutralized to pH 7 and assayed. The
26 micro bicichoninic acid (BCA) method was utilized with

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1 both lactic acid and glycolic acid blanks and compared
2 to bovine serum albumin (BSA) standard and results
3 expressed as percent by weight.

4 Moisture Content. One hundred and fifty mgm of
5 CFA/II microspheres were dissolved in 3 ml of
6 acetonitrile by sonication for 3 hours. One ml sample
7 was injected into a Karl Ficher titrimeter and triter
8 reading observed was recorded and acetonitrile blank
9 was subtracted to determined percent water content.

10 Acetonitrile and Heptane Residuals. Ten mgm of
11 CFA II microspheres were dissolved in 1 ml DMF then
12 analysed using gas chromatography and comparing peak
13 heights to external standards of either acetone or
14 heptane diluted in DMF with 10 mgm of blank
15 microspheres. The results are expressed as percent by
16 weight.

17 Microbial load. One hundred mgm of CFA/II
18 microsphere(single dose) are suspended in 2 ml of
19 sterile saline than poured into 2 blood agar plates (1
20 ml each). All colonies are counted and identified
21 after 48 hours in culture at 37° C and expressed as
22 total number. Similiar amount of microspheres is in
23 0.25 ml aliquots poured onto 4 different fungal culture
24 plates (Sabharwal, casein peptone agar with
25 chloramphenicol, brain heart infusion agar with

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1 chloramphenol and genimycin or chloramphenicol alone)
2 and cultured at 30° for 5 weeks and the colonies
3 counted & identified and expressed as total number.

4 CFA/II Release From Microsphere Study. Thirty
5 mgm samples in triplicate were placed in 2 ml conical
6 upright microcentrifuge tubes containing 1 ml of PBS at
7 pH 7.4. The tubes were capped and kept immerized in a
8 water bath maintained at 37° C with constant agitation.
9 The samples were withdrawn at 1, 3, 6, 8, 15 and 22
10 hour time intervals by centrifuging the sample tubes
11 for 5 minutes at the maximum speed of bench top
12 centrifuge. The release medium was collected through a
13 5 um nylon screen for CFA/II protein analysis using the
14 micro BCA method and comparing results to BSA standard
15 and expressing results as percent cumulative release of
16 CFA/II.

17 General Safety Test. Two doses of one hundred
18 mgm CFA/II microspheres were suspended by sonication
19 for 5 minutes in 3.1 mls of sterile vaccine diluent
20 consisting of injectable saline containing 0.5%
21 Polysorbate 60^R N.F., 0.03 ml were injected
22 intraperitoneally into each of 2 mice and 3 mls were
23 administered by gastric lavage to each of 2 guinea
24 pigs. The animals were weighed both before and at 7
25 days following the vaccine administration. All animals
26 were observed daily for any signs of toxicity.

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1 Rabbits. 1.5-2 kilogram male specific pathogen
2 free New Zealand white rabbits, obtained from closed
3 colony maintained at the National Institute of Health,
4 Bethesda, MD. They were selected for study if they did
5 not have measurable serum antibodies at 1:2 dilution to
6 CFA/II antigens by ELISA and were not colonized by E.
7 coli as determined by culture of rectal swabs.

8 Intra-Muscular Immunization of Rabbits and ELISA.

9 Two Rabbits were immunized with CFA/II microsphere
10 vaccine at 25 ug protein in two different sites intra-
11 muscularly on day 0. Sera were obtained from all
12 animals before immunization on day 0 and days 7 and 14.
13 The sera were tested by ELISA for IgG antibodies to
14 CFA/II antigen and individual coli surface (CS)
15 proteins CS3 and CS1. ELISA plates were coated with 3
16 ug/ml of either CFA/II antigen, CS3 or CS1 protein (150
17 ul/well) and incubated with 150 ul/well of PBS with
18 0.1% BSA for four hours at room temperature. The PBS
19 with 0.1% BSA is washed out with PBS and 100 ul/well of
20 different dilutions of each rabbit serum in triplicate
21 was added to the plates. The dilutions ranged from
22 undiluted to 1:1,000,00. The plates were incubated
23 with the sera for 3 hours at 37° C. The sera were
24 washed out with PBS and then horse radish peroxidase-
25 conjugated goat anti- rabbit IgG was added to the
26 plates at a 1:1000 dilution (100 ul/well). The plates

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1 were incubated for 1 hour at room temperature with the
2 peroxidase conjugate. The conjugates were washed out
3 of the plates with PBS and 100 ul/well of an ABTS
4 substrate solution (Kikegaard and Perry Laboratories)
5 was added to each well in the plates. The plates were
6 read using the ELISA reader (Dynatech Laboratories MR
7 580) at a wave length of 405 nm after 15 minutes.
8 The results are measured and expressed as antibody
9 titers.

10 Intra-duodenal Vaccination of Rabbits. Rabbits
11 (N=5) were vaccinated with CFA/II microspheres
12 containing either 25 or 50 ug of protein suspended in 1
13 ml of PBS containing 0.5% Polysorbate 60[®] on day 0 and
14 7 by sonication. The microspheres were injected
15 through an Olympus BF type P10 bronchoscope into the
16 duodenum of the rabbits following sedation with an
17 intra muscular injection of ketamine HCl (50 mgm
18 I.M.) (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA)
19 and Lylazine (10 mgm I.M.) (Rompom Malay Corporation,
20 Shnane, KS). The endoscope was advanced ready under
21 direct vision into the stomach which was insufflated
22 with a 50 ml bolus of room air via a catheter passed
23 through the biopsy channel. The catheter was advanced
24 through the pylorus 3-4 cm into the duodenum and the
25 microsphere suspension in 1 ml of PBS was injected,
26 followed by a 9 ml flush of PBS and removal of the air

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1 bolus. The rabbits were sacrificed by chemical
2 euthanasia at day 14.

3 Anti-CFA/II Stimulated Lymphocyted

4 Transformation. The Peyer's Patchs were removed and
5 cell suspension obtained by teasing and irrigation with
6 a 20 guage needle and syringe. The cells were placed
7 in 2 ml of media at a concentration of 2.5×10^6
8 cells/ml for each well of a 24 well plate. These cells
9 were challenged separately with BSA and the CFA/II
10 antigen at doses of 500, 50 and 5 ng/ml in triplicate
11 wells. The plates were incubated at 37° C with 5% CO₂.
12 On day 4 the cells were mixed while still inside the
13 wells and 100 ul were transferred into each of 4 wells
14 in a 96 well flat bottom microculture plate. Thus, the
15 challenge at each antigen dose represented by 3 wells
16 in the 24 well plate is now represented by 12 wells in
17 the 96 well plate. After the cells have been
18 transferred, each well is pulsed with 20ul of 50 uCi/ml
19 tritiated thymidine. These pulsed plates were
20 incubated for 6 hrs then harvester with 96 Mach II Cell
21 harvested (Tourtec, Inc.). The lymphocyte
22 proliferation was determined by the tritriated
23 thymidine incoporation measured in kilo counts per
24 minute (Kcpm) using the 1205 Beta Plate Liquid
25 scintillation counter (LKB, Wallac, Inc.). The results

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1 are expressed as mean Kcpm \pm SD and compared to media
2 controls.

3 Anti-CFA/II Antibody Secreting B Cells. Spleen
4 cells were obtained from immunized rabbits on day 14
5 following intra-duodenal immunization with CFA/II
6 microsphere vaccine. The cells were placed in 96 well
7 round bottom microculture plate at a final
8 concentration of 6×10^5 cells/well and incubated for
9 0, 1, 2, 3, 4 and 5 days at 37° C with 5 CO₂. 96 well
10 flat bottom microculture plates were coated with 3
11 ug/ml of CFA/II antigen overnight blocked with PBS with
12 0.05% Polysorbate 60[®]. On the harvest days, the cells
13 were gently flushed out of the wells of the round
14 bottom plates and transferred to the corresponding well
15 in the antigen coated, 96 well flat bottom microculture
16 plates to be tested for the presence of antibody
17 secreting cells using ELISPOT technique. The plates
18 were incubated with the cells overnight at 4° C. The
19 cells were then washed out of the flat bottom plates
20 with PBS, and 100 ul/well of horserudish-peroxidase
21 conjugated, goat anti-rabbit total antibody (IgM, IgG,
22 and IgA) at a 1:1000 dilution were added to the plates.
23 The Plates were incubated for 1 hour at room
24 temperature, at which time, the conjugate was washed
25 out of the plates with PBS. 0.1 mgm of agarose was
26 dissolved in 10 ml of PBS by boiling. After the agar

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1 solution cooled but not hardened, 6 mgm of 4-chloro-
2 naphthol, 2 mls of methanol and 30 ul of hydrogen
3 peroxide were added to make the substrate solution.
4 The solution was placed into the flat bottom plates
5 (100 ul/well) and the plates were held at 4°C overnight
6 so the agar could harden. The number of browish spots
7 per 15 wells (total of 9×10^6 spleen cells) was
8 counted and represents the number of antibody secreting
9 cells per 9×10^6 spleen cells.

10 Pathological Evaluation. Rabbits were euthanized
11 by parenteral overdose of sodium pentobarbital and were
12 subjected to complete necropsy. Sample of tissue
13 including small and large intestine with gut associated
14 lymphoid tissue, spleen, mesenteric and mediastinal
15 lymph nodes, lung, trachea, liver and kidney were fixed
16 by immersion in 10% neutral buffered formalin. Tissues
17 were routinely processed for light microscopy and
18 embedded in paraffin. Five micron thick sections were
19 stained with hematoxylin and eosin.

20 Statistical Analysis. The paired student t-test
21 was used to determine p values.

22 RESULTS

23 Particle Size Distribution. The results of size
24 frequency analysis of 150 randomly chosen microspheres
25 are shown in (Figure 29). The particle size

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1 distribution is plotted in % frequency against particle
2 size in diameter (size) expressed in μm . The average
3 number frequency diameter is 4.6 μm . The average volume
4 frequency diameter is 4.6 μm . The percent volume
5 between diameters of 5-10 μm is 63% and the percent
6 volume less than 10 μm diameter is 88%.

7 Scanning Electron Microscopy. The microspheres
8 are seen in (Figure 30) which is a scanning electron
9 photomicrograph. Nearly all the microspheres are less
10 than 10 μm as compared to the 5 μm bar. Also the
11 surfaces of the microsphere are smooth and demonstrate
12 lack of pores.

13 Protein Content. The protein loads of the
14 individual batches are the following: K62A8, 1.16% \pm
15 0.10 SD; K63A8, 1.023% \pm 0.17SD; K64A8, 1.232% \pm 0.13
16 SD; and K65A8, 0.966% \pm 0.128 SD. The mean average
17 protein load is 1.16% \pm 0.15 SD. The protein load of
18 the CFA/II microsphere vaccine in the final dose vial
19 is the following: Lot L74F2, 1.175% \pm 0.17SD.

20 Moisture Content. The CFA/II microsphere vaccine
21 (Lot 74F2) percent water content was found using the
22 Karl Fischer titrimeter method to be 2.154% using
23 triplicate samples.

24 Acetonitrile and Heptane Residuals. The
25 acetonitrile residuals of the 4 individual CFA/II
26 microsphere batches are the following: K62A8, <0.1%;

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1 K62A8, <0.1%, K64A8, <0.1%; and K65A8, <0.1%. The
2 acetonitrile residual of the CFA/II microsphere vaccine
3 in the final dose vial is the following: Lot L74F2,
4 $0.07 \pm 0.05\%$. The heptane residual of the 4 individual
5 CFA/II microsphere batches are the following: K62A8,
6 1.9%; K63A8, 1.4%; K64A8, 1.6% and K65A8, 1.6%.
7 Following pooling in heptane and subsequent drying, the
8 heptane residual of the CFA/II microsphere vaccine in
9 the final dose vial is the following: Lot L74F2, $1.6 \pm$
10 0.1% .

11 Microbial load. One hundred milligrams (a single
12 dose) of CFA/II microsphere vaccine (Lot L74F2) in the
13 final dose vial was suspended in a 2 ml of sterile
14 saline and 1 ml poured onto a blood agar culture plate
15 x 2. Twenty two colonies grew after 48 hours of
16 culture and 21 were identified as coagulase negative
17 staphylococcus and 1 as a micrococcus species. All these
18 bacteria are considered to be nonpathogenic to humans.
19 An additional 100 mgms of CFA/II microsphere vaccine
20 (Lot L74F2) were suspended in 2 ml of sterile saline
21 and 0.25 ml poured onto four different fungal culture
22 agars and cultered for 5 weeks. Three fungal colonies
23 grew and each was identified as A. glaucus.

24 CFA Release From Microsphere Study. Three thirty
25 mgm samples were incubated each in 1 ml of PBS, pH 7.4

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1 at 37° C for 0, 1, 3, 6, 8, 15 and 22 hours. The
2 supernates were removed and replaced at these times.
3 The protein content was determined for each supernate
4 sample and the results are seen in (Figure # 31). The
5 results are plotted as percent release of CFA/II
6 against time in hours. An average of 8% of CFA/II is
7 released at one hour rising to 20% at 8 hours then a
8 slower release to 25% at 22 hours.

9 General Safety Test. Two one hundred milligrams
10 (a single dose) of CFA/II microsphere vaccine in the
11 final dose vials were suspended in 3.1 mls of the
12 sterile diluent consisting of 0.85 N saline prepared
13 for injection plus Polysorbate 60^R at 0.5%. Two Swiss
14 mice (16.5 gm) were injected intraperitoneally with
15 0.03 mls and two Hartley guinea pigs (350 gm) were
16 administered by gastric lavage 3.0 mls.

17 None of these animals displayed any signs of
18 toxicity for 7 days. The mice gained an average of
19 2.3 gms and the guinea pigs gained an average, of 43
20 grams. The CFA/II microsphere vaccine therefore
21 passed the general safety test.

22 Serum IgG Antibody Responses. Two rabbits were
23 immunized in two separate sites intra-muscularly with
24 25 ug of protein of CFA/II microsphere vaccine (Lot
25 L74F2) in the final dose vial. Sera samples were
26 obtained before and 7 and 14 days following

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1 immunization. The IgG antibody titers to CFA/II CSI
2 and CS3 protein were determined using ELISA and the
3 results seen in (Figure 32). The results are expressed
4 as mean antibody titers against the different antigens
5 at 0, 7 and 14 days. High antibody titers greater than
6 1000 were seen at 7 days to both CS1 and CS3 protein
7 which rose to greater than 10,000 by day 14. The
8 individuals titers to CFA/II are seen in (Figure 33).
9 Rabbit 109 developed an antibody titer of 1,000 by day
10 7 rising to 3,000 by day 14. Rabbit 108 had a log
11 higher rise at day 7 and 2 log higher rise at day 14
12 being 3×10^4 at day 7 going to 1×10^5 at day 14.

13 Anti-CFA/II Stimulated Lymphocyte Transformation.

14 Five rabbits were immunized intra-duodenally with
15 CFA/II microspheres containing either 25 ug of protein
16 (human dose equivalent) or 50 ug of protein on days 0
17 and 7 and then sacrificed on day 14. The Peyer's patch
18 lymphocytes were challenged in vitro with CFA/II
19 antigen, BSA media and alone. The lymphocyte
20 transformation was determined by tritiated thymidine
21 incorporation. The results of the high dose
22 immunization are seen in (Figure 34). The results are
23 expressed as Kcpm against antigen dose. No response to
24 BSA or media control is seen in any of the five
25 rabbits. All rabbits responded by lymphocyte

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1 transformation in a dose dependent manner to the
2 CFA/II.

3 The highest dose responses were 3-10X's the media
4 control are highly significant with a p value of
5 <0.002. The results of the 5 rabbits receiving the low
6 dose immunization are seen in (Figures 35). Rabbit #80
7 gave no response probably due to poor Peyer's patch
8 cell population which did not respond were to
9 Conconavallin A mitogenic stimulation either. The
10 remaining 4 rabbits gave positive responses with the
11 high CFA/II dose response being 2-8x media control and
12 highly significant with p values of <0.009. Again no
13 response were seen to BSA compared to the media cont

14 Anti-CFA/II Antibody Secreting B-Cells Five
15 rabbits immunized intraduodenally with CFA/II
16 microsphere containing 50 ug of CFA/II protein at days
17 0, 7 than sacrificed at day 14 were studied. The spleen
18 cells were placed into microculture then ELISPOT
19 forming B-Cells secreting specific anti CFA/II antibody
20 determined at days 0, 1, 2, 3, 4 and 5. The results
21 are seen in (Figure 36) and expressed as # of antibody
22 secreting cells per 9×10^6 spleen cell against culture
23 days. Positive responses were seen in all 5 rabbits on
24 days 2-5. Days of maximum responses occurred on day 3
25 for rabbits 65 and 66; day 4 for rabbit 85; and day 5
26 for rabbits 83 and 86. The responses are highly

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1 significant being 7-115 times higher than the 1-2 cells
2 seen on all days in 4 control rabbit (67, 69, 72, 89)
3 (Figure 37). Here is a composite graph expressing the
4 mean counts \pm ISD for all days of culture.

5 Pathological Evaluation. A consistent finding in
6 the spleens of all rabbits both the 25 and 50 ug
7 protein dose groups was minimal to mild diffuse
8 lymphocytic hyperplasia the periarteriolar lymphatic
9 sheaths (T cell dependent areas). Two of five rabbits
10 of the 50 ug dose group (#83 and #86) also had mild
11 lymphocytic hyperplasia of splenic follicular (B cell
12 dependent) areas. The three rabbits in an untreated
13 control group had histologically normal spleens.

14 Reactive hyperplasia of mesenteric lymph nodes
15 was often seen in vaccinated rabbits. Two of five
16 rabbits in the 25 ug dose equivalent group (#83 and
17 #86) also had minimal to mild lymphocytic hyperplasia
18 of cortical follicular (B cell dependent) areas. The
19 mesenteric lymph nodes of the other vaccinated rabbits
20 and of the untreated control rabbits were within normal
21 limits. Incidental or background lesions found in one
22 or more rabbits of all three group were acute minimal
23 to mild pneumonia and foreign body microgranulomas of
24 the cecal gut associated lymphoid tissue.

25 Discussion

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1 McQueen et al (33) has found that the AF/R1 adhesin of
2 rabbit diarrheagenic Escherichia coli (RDEC-1)
3 incorporated into biodegradable microspheres could
4 function as a safe and effective oral intestinal
5 vaccine in the rabbit diarrhea model. The AF/R1 was
6 incorporated into poly D,L-lactide-co-glycolide)
7 microspheres and administered intraduodenally. Jarboe
8 et al (34) reported that

9 Peyer's patch cells obtained from rabbits
10 immunized intra-duodenally with AF/R1 in microspheres
11 responded with lymphocyte proliferation upon in vitro
12 challenge with AF/R1. This early response at 14 days
13 gave a clear indication as to the immunogenicity of E.
14 coli pili contained within the polymer microspheres.

15 In developing an effective oral vaccine against
16 enterotoxigenic E. coli, CFA/II pili given as an oral
17 vaccine was found to be ineffective. The CFA/II pilus
18 proteins were found to be rapidly degraded when treated
19 with 0.1M HCl and pepsin conditions mimicking those
20 contained in the stomach (27). The CFA/II was found to
21 be immunogenic when given in high doses intra-
22 intestinally producing intestinal secretory IgA
23 antibodies (26).

24 The CFA/II vaccine has now been incorporated into
25 poly(D,L lactide-co-glycolide) microspheres under Good
26 Manufacturing Practices and tested under Good

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1 Laboratory Practices. The microspheres, are spherical,
2 smooth surfaced and without pores. The majority (63%)
3 are between 5-10 um in diameter by volume. This size
4 range has been suggested to promote localization within
5 the Peyer's patch in mice and perhaps enhance local
6 immunization (29-32). The protein content being 1.174%
7 is close to 1% which was the goal of the vaccine
8 formulation. One percent was chosen because 0.62% was
9 the core loading of the AF/R1 microspheres which were
10 effective. Also a small precentage perhaps 1-5% (35)
11 is anticipated to be taken up from the intestine, a
12 higher protein content would lead to considerable loss
13 of protein.

14 The organic residuals are of course a concern.
15 Heptane exposure would be 1.7 mgm per vaccine dose.
16 This is compared to the occupational maximum allowable
17 exposure of 1800 mgm/15 min. Therefore, the heptane
18 contained with the CFA/II microsphere vaccine appears
19 to be a safe level. The acetonitrile is very low - 0.1
20 mgm per vaccine dose. The human oral TDLO is 570
21 mgm/Kg (any non letheal toxicity). Therefore, the
22 acetonitrile contained with the CFA/II microsphere
23 vaccine appears to be at a safe level. The CFA/II
24 vaccine was produced under sterile conditions.
25 However, the process of incorporation of the desalted
26 CFA/II vaccine into the polymer microsphere batches and

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1 subsequent pooling and loading final dose vials was
2 done in a clean room as for any oral medication. It
3 was expected and found that there was be a microbial
4 load. The guide used was the World Health Organization
5 (WHO) Requirements of Thyphoid Vaccine (Live
6 Atttaruated, Ty 21a oral). Two hundred non pathogenic
7 bacteria are allowed as well as 20 fungi per dose. The
8 CFA/II microsphere vaccine is well under these
9 requirements having only 22 non-pathogenic bacteria and
10 3 fungi per dose.

11 The general safety test was also patterned after
12 the WHO requiremets for the TY, 21a oral vaccine in
13 that the CFA/II microsphere vaccine was give by gastric
14 lavage to the guinea pigs. Both mice and both guinea
15 pigs demonstrated no toxicity & gained weight over the
16 7 day test clearly indiciating the innoccuos nature of
17 this vaccine by passing this safety test.

18 The CFA/II microsphere vaccine (Lot74F2) is
19 immunogenic giving high titer serum IgG antibody
20 responses as early as 7 days following intra muscular
21 injection in rabbits. This test will be used as
22 potency test for future lots of the CFA/II microsphere
23 vaccine. Slighly higher antibody titers were seen
24 towards the CS3 pilus protein and this may reflect that
25 CS3 accounts for 90% of the protein in the CFA/II and
26 CS1 10% (36).

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1 The CFA/II microsphere vaccine was also
2 immunogenic following intra-duodenal administration to
3 rabbits. The highest lymphocyte proliferative
4 responses from Peyer's patch cells were seen with the
5 lower 25 ug dose. This is the human equivalent dose
6 and suggests that higher doses of antigen in polymer
7 microspheres may attenuate, this immunological response.

8 The antibody secreting B-cells demonstrated in
9 the rabbit spleen at 14 days is a clear indication that
10 B-cells have been immunized. They may represent
11 resident B-cells immunized in the spleen or B-cells
12 immunized at the level of the Peyer's patches and are
13 migrating through the spleen to return to the
14 intestinal mucosal lamina propria (1-3). The delay of
15 several days before secreted antibody is detected
16 suggests either maturation is required of the B-cells
17 or that down regulation may be present initially and
18 lost with time in culture.

19 Further evidence of immunization by the CFA/II
20 microsphere vaccine given intra-duodenally is
21 demonstrated by the lymphatic hyperplasia in the spleen
22 seen to a greater extent in the rabbits receiving the
23 lower dose 5/5 compared to 2/5 of the rabbits receiving
24 the higher 50 ug protein dose. On the other hand,
25 greater T-cell dependent area lymphocytic hyperplasia in
26 the mesenteric lymph nodes were seen in rabbits

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1 receiving the higher 50 ug dose, 4/5 compared to 2/5.
2 These changes are most likely due to the vaccine since
3 similar changes were not seen in three untreated
4 control rabbits. Also no abnormal pathological changes
5 attributable to the vaccine were seen.

6 The CFA/II BPM vaccine has undergone pre-clinical
7 evaluation and has been found safe and immunogenic.
8 This vaccine is ready for clinical Phase I safety
9 testing following FDA's IND approval.

10 PHASE IV

11 In sum, alum precipitation, vaccination regimen
12 and controlled delivery by microencapsulation were
13 studied to determine what criteria must be satisfied to
14 provide a protective immune response to hepatitis B
15 surface antigen (HBsAg) after a single injection of
16 vaccine. In mouse studies, the 50% effective dose
17 (ED₅₀) for the alum precipitated Heptavax B vaccine
18 (Merck, Sharp and Dohme) was 3.8 ng when administered
19 in a 3 injection regimen, but was 130 ng when one
20 immunizing dose was used. Antigen release studies
21 revealed that HBsAg is bound tightly to the alum,
22 indicating that the antigen remains in situ until
23 scavenged by phagocytic cells. the ED₅₀ with a 3 dose
24 regimen of aqueous HBsAg was 180 ng, as opposed to over
25 2000 ng for daily injections of low doses for 90 days
26 and 240 ng for a regimen that employed initially high

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1 doses that decreased geometrically at 3 day intervals
2 over 90 days. The ED₅₀ was 220 ng for a single dose
3 regimen of HBsAg microencapsulated in poly (DL-lactide-
4 co-glycolide) in a form that was too large to be
5 phagocytized and had an antigen release profile similar
6 to that achieved with the geometrically decreasing
7 regimen of doses. This indicates that single injection
8 of microencapsulated immunogens can achieve similar
9 effects in vivo to those achieved with multiple dose
10 regimens. For HBsAg the effect to be achieved appears
11 to be 3 pulses of particulate immunogens that can be
12 scavenged by phagocytes.

13 INTRODUCTION

14 A major disadvantage of inactivated vaccines
15 lies in their inability to confer lasting immunity.
16 Due to rapid elimination from the body, multiple doses
17 and boosters are usually required for continued
18 protection³⁷. Alum adjuvants, achieving their effects
19 by mechanisms of antigen presentation and sustained
20 antigen release³⁸, have been used successfully to
21 increase the potency of several inactivated vaccines
22 including those against tetanus, anthrax, and serum
23 hepatitis^{39,40}. Though useful, alum preparations are
24 deficient in several aspects. Control over quantity
25 and rate of antigen release is limited, often resulting
26 in a continued requirement for immunization schedules

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1 consisting of multiple injections given over a period
2 of several months to years. Alum adjuvants are also
3 non-biodegradable and thus remain within the body,
4 serving as a nidus for scar tissue formation³⁸ long
5 after they have served their function.

6 Protracted, multiple immunization schedules are
7 unacceptable during massive mobilization and deployment
8 of troops. Changing global disease patterns and
9 deployment of new biological warfare agents by enemy
10 forces require flexibility in the number and types of
11 vaccine antigen administered to soldiers departing for
12 combat. Any immunization schedule requiring completion
13 during engagement in non-linear combat would compromise
14 this flexibility and place an unreasonable burden on
15 our health care delivery system.

16 The main objective of this study was, therefore,
17 to develop a biodegradable, controlled-release adjuvant
18 system capable of eliminating the need for multistep
19 vaccination schedules. This investigation was designed
20 to : (1) determine in an animal model hepatitis B
21 vaccine release rate characteristics desirable for
22 single-step immunization, (2) incorporate those release
23 rate characteristics into a one-step biodegradable
24 poly(DL-lactide-co-glycolide) (DL-PLG) microencapsulated
25 hepatitis B surface antigen (HBSAg) vaccine, and (3)
26 conduct an in vivo trial comparing the effectiveness of

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1 this single-step vaccine against the conventional
2 three-step hepatitis vaccine currently employed⁴¹. The
3 results were intended to provide the foundation for
4 further development of single step vaccines against
5 hepatitis and other militarily significant diseases⁴².

6 MATERIALS AND METHODS

Vaccine potency assay. Due to its availability, compatibility with cage mates, and potential application to the study of hepatitis B vaccine⁴³, the female Walter Reed (ICR) strain mouse was used. A hepatitis B vaccine potency assay for comparing the six-month immunization schedule currently in use⁴¹ with that of a single-step immunization by sustained antigen release was established according to the following protocol: Specimens for baseline antibody titers were collected from twenty mice by exsanguination. Immediately prior to exsanguination, all mice employed in this and other exsanguination procedures in these studies were anesthetized with a 0.1 ml injection of V-Pento. Groups of 12 mice were then immunized according to a schedule consisting of either 0.25 ug, 0.025 ug, 2.5 ng, 0.25 ng, 2.5 pg, or 0.25 pg Heptavax B vaccine (HBV) administered in 50 microliter volumes subcutaneously (s.c.) at the beginning and end of the first, and end of the second month of the protocol. Antibody responses to the vaccine were monitored

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1 immediately before the third injection and
2 approximately one month after the third injection.
3 Specimens for antibody determination were collected by
4 exsanguination of seven anesthetized mice from each
5 group and assayed along with the baseline samples by
6 the Abbott Ausab radioimmunoassay. Percent
7 seroconversion verses micrograms vaccine employed with
8 calculated by the method of Reed and Muench⁴³. These
9 data were employed to establish a mouse vaccine potency
10 assay calibrated to detect differences between Heptavax
11 B and other forms of hepatitis b vaccine.

12 In vitro antigen release rate from Heptavax B
13 vaccine. Antigen release from aluminum hydroxide
14 adjuvant in HBV was measured by pumping 2 cc per hour
15 of 1:20,000 thimerosal in saline at 4°C across a 0.2 u
16 pore diameter Acrodisc filter apparatus containing 20
17 ug of vaccine. The effluent, collected by a Gilford
18 fraction collector, was assayed periodically over
19 several weeks for protein by UV absorption at 280 nm on
20 a Beckman model 25 double beam spectrophotometer, and
21 for HBsAg by the Abbot Ausria II radioimmunoassay made
22 quantitative by using HBsAg standards supplied by Merk,
23 Sharp, and Dohme. Accuracy of the HBsAg standards were
24 verified by Biuret protein determination and by UV
25 absorbance at 215 nm and 225 nm⁴⁴. Nonspecific antigen
26 retention on the Acrodisc filter was assessed by

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1 measuring percent recovery of a known quantity of
2 HBsAg. Spontaneous degradation of vaccine antigen was
3 monitored by comparing daily rations of antigen to
4 total protein detected in the effluent.

5 Evaluation of HBsAg stability. These studies
6 were designed to characterize the stability of the
7 aqueous antigen to the various physical conditions
8 employed in the microencapsulation process. Conditions
9 tested included lyophilization with reconstitution in
10 distilled water, cyclohexane, methylene chloride,
11 chloroform, methyl alcohol, acetone, iso-octane,
12 hexane, acetone, pentane, or heptane; irradiation while
13 lyophilized; and, exposure to elevated temperatures.
14 Samples exposed to organic solvents were first
15 lyophilized, reconstituted with the test solvent,
16 evaporated to dryness under nitrogen at room
17 temperature and reconstituted with distilled water.
18 Test samples were compared against untreated controls
19 by assaying serial dilutions of each with the Abbot
20 Ausria II procedure and comparing the plots of counts
21 per minute verses dilution.

22 Assessment of the effect of antigen release rate
23 on vaccine potency. Three regimens simulating patterns
24 of free HBsAg release that could be achieved by
25 microencapsulation were contrasted with the three
26 monthly dose regimen of Heptavax B for immunizing mice.

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1 To do so, 24 ICR mice were divided into groups and
2 vaccinated as indicated below. Seven mice from each
3 subgroup were exsanguinated at the end of the second
4 and third months of the experiment. The sera were
5 separated and assayed for specific antibody response to
6 HBsAg by Abbot Ausab procedure.

7 HV regimen a: 14 mice/treatment receiving 3 s.c.
8 injections of 250, 25, 2.5 or 0.25 ng doses of HBV a
9 month apart.

10 HBsAg regimen a: 14 mice/treatment receiving 3
11 s.c. injections of 250, 25, 2.5 or 0.25 ng doses of
12 aqueous HBsAg a month apart.

13 HBsAg regimen b: 14 mice/treatment receiving
14 total doses of 750, 75, 7.5 or 0.75 ng of aqueous HBsAg
15 over 3 months by s.c. injections of ZX_y ng at 3 day
16 intervals, where Z is the total dose, y is the
17 injection number, and X is the fraction indicated on
18 the graph in Fig. 1 minus the fraction for the previous
19 injection.

20 HBsAg regimen c: 14 mice/treatment receiving
21 daily s.c. injections of 8.33, 0.833, 0.0833 or 0.00833
22 ng of aqueous HBsAg for 3 months.

23 Microencapsulation in DL:PLG. Microencapsulated
24 immunogens were fabricated by Southern Research
25 Institute, Birmingham, AL. DL-PLG polymers were
26 synthesized from the cyclic diesters, DL lactide and

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1 glycolide, by using a ring-opening melt polymerization
2 catalyzed by tetraphenyl tin⁴⁵. The resulting polymer
3 was dissolved i methylene chloride, filtered free of
4 insoluble contaminants and precipitated in methanol.
5 Lactide-co-glycolide mole ration of the product was
6 determined by nuclear magnetic resonance spectroscopy.
7 Encapsulation of HBsAg in DL:PLG polymer was achieved
8 by an organic phase separation process⁴⁶. Microcapsules
9 of the desired size (approximately 100 micron diameter
10 in these studies) were isolated from each batch by wet
11 sieving with hexane through standard mesh stainless
12 steel sieves and then dried for 24 hours in a vacuum
13 chamber maintained at room temperature.

14 In vitro analysis of encapsulated antigens.

15 Integrity of encapsulated antigen was assessed by
16 comparing the antigen to total protein ratios present
17 in microcapsule hydrolysates with those obtained from
18 suspensions of pure unencapsulated antigen. Centrifuge
19 tubes containing 1 ug of either microencapsulated or
20 pure vaccine antigen in 1 ml saline were incubated at
21 4°C with shaking. Samples were collected at weekly
22 intervals by interrupting the incubation, sedimenting
23 the contents of the tubes by centrifugation and
24 withdrawing the supernates. Sediments were resuspended
25 in 200 microliters of saline and supernates were
26 assayed for HBsAg by the Abbott Ausria II

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1 radioimmunoassay. The HBsAg standard described earlier
2 in this report was used as the calibrator. Antigen
3 destruction due to the encapsulation procedure was
4 monitored by a comparison between the antigen assayed
5 from the hydrolysate and from the untreated antigen
6 control.

7 Assessment of the potency of DL:PLG
8 microencapsulated HBsAg for immunizing ICR mice when
9 used alone and in combination with Heptavax B vaccine.
10 HBsAg loaded microcapsules that had been fabricated by
11 Southern Research Institute to release the majority of
12 their HBsAg load within 40 to 50 days were serially
13 diluted in 10-fold steps by mixing the dry, loaded
14 capsules with blank placebo capsules of similar size
15 and composition. The resulting stock and diluted
16 microcapsule preparations were placed onto lyophilizer
17 when not in use in order to assure minimum spontaneous
18 degradation prior to injection. On the day of
19 injection, a predetermined weight of microcapsules or
20 placebo-diluted microcapsules was added to each
21 syringe. Immediately prior to injection either one or
22 two ml of injection vehicle (2 wt % carboxymethyl
23 cellulose and 1 wt % Tween 240 in water, Southern
24 Research Institute) were drawn into the microcapsule-
25 loaded syringes, mixed and injected. All mice were
26 vaccinated s.c. as indicated below:

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1 Group 1: 14 mice/treatment receiving 25, 25, 2.5,
2 0.25 or 0.925 ng HBV.

3 Group 2: 14 mice/treatment receiving 1000, 250,
4 25 or 2.5 ng aqueous HBsAg with Bovine Serum Albumin
5 (BSA).

6 Group 3: 7 mice receiving 1600 ng
7 microencapsulated HBsAg (HBsAg) plus 0.25 ng HBV and 14
8 mice/treatment receiving 160, 16, 1.6 or 0.16 ng HBsAg
9 plus 0.25 ng HBV.

10 Group 4: 7 mice receiving 1600 ng HBsAg plus 2.5
11 ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or
12 0.16 ng HBsAg plus 2.5 ng HBV.

13 Group 5.: 7 mice receiving 1600 ng HBsAg plus 25
14 ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or
15 0.16 ng HBsAg plus 25 ng NBV.

16 Group 6: 7 mice receiving 2500 ng HBsAg and 14
17 mice-treatment receiving 250, 25, 2.5 or 0.25 ng HBsAg.
18 Fifty-three days after receiving the above injections,
19 the mice were anesthetized with an 0.1 cc injection of
20 V-Pento and exsanguinated. Blood samples were allowed
21 clot and the sera were separated by centrifugation.
22 The serum samples were assayed for antibody to HBsAg by
23 the Abbott Ausab procedure.

24

RESULTS

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1 Heptavax B vaccine potency. As can be seen from
 2 Table 4, the total dose of vaccine which produced
 3 seroconversion in 50% of

4 TABLE 4. Potency of Heptavax B vaccine in ICR mice.

5								
6	No.	ng Heptavax B per Injection						
7	ED ₅₀							
8	Inj.	250	25	2.5	.25	.025	.0025	.00025
9	ng							
10								
11	2	5/5	4/4	3/6	2/6	0/5	1/4	0/4
12	1.7							
13	3	6/6	6/6	4/6	1/6	0/6	1/6	1/6
14	2.0							
15								

16 * Number positive seroconversions per number
 17 vaccinated.

18 The vaccinated mice (ED₅₀) for HBV was approximately 2
 19 ng, whether the vaccine was given in 2 or 3 injections.

20 In vitro antigen release rate from HBV. HBsAg
 21 release from the 20 ug of Heptavax was not detected in
 22 any of the 21 fractions of saline collected from the
 23 Acrodisc polycarbonate filter over a 30 day period.
 24 The lower limit of detection for the Abbott Auria II
 25 assay employed was approximately 4.8 ng/ml. The

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1 Acrodisc filter used in the antigen release study was
2 back-washed with 10 mls normal saline. Quantitation of
3 the HBsAg present within this back-wash eluent revealed
4 the presence of the original 40 ug of Heptavax vaccine
5 which had been loaded into the filter at the start of
6 the experiment. This is the concentration which one
7 would expect to obtain if there had been no
8 deterioration of the original 40 ug/ml HBsAg loaded
9 onto the filter, none of the antigen eluted from the
10 alum adjuvant, and none of the vaccine had adsorbed
11 onto or passed through the filter.

12 Evaluation of antigen stability. Considerable
13 effort was expended in assessing the effects of
14 physical conditions on the antigenicity of HBsAg to
15 insure that the conditions used for microencapsulation
16 would not cause serious degradation of the immunogen.
17 Since microencapsulation must be performed on dried
18 materials which are suspended in organic solvents, the
19 HBsAg, which was provided as a solution, had to be
20 lyophilized. Initial attempts at lyophilizing HBsAg in
21 normal saline resulted in a total loss of detectable
22 antigen within samples. Dilution of the HBsAg sample
23 1:10 in distilled water prior to freezing resulted in
24 reservation of nearly 100% of the antigen detectable in
25 the original sample. Studies of antigen stability at
26 elevated temperature revealed that HBsAg may be heated

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1 to 50°C for up to one hour without appreciable loss of
2 antigen. The studies involving exposure of lyophilized
3 antigen to organic solvents indicated that iso-octane and
4 hexane had minimal effects on antigenicity, but that
5 95% to 100% of antigenicity was lost upon exposure to
6 either methylene chloride, chloroform, cyclohexane, or
7 methyl alcohol. Moderate antigen loss occurred in the
8 presence of acetone, pentane and heptane. As a result
9 of these studies, hexane was chosen as the solvent for
10 microencapsulation.

11 Assessment of the effect of antigen release rate
12 on vaccine potency. The results (Table 5) indicated
13 that immunogen formation (i.e., the alum adjuvant of
14 Heptavax B) had far more

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TABLE 5. Effect of immunogen formulation and vaccination regimen on potency for immunizing ICR mice.

Immunogen		ng Total Dose HBsAg					ED ₅₀
Formulation	Regiment	750	75	7.5	.75	ng	
Heptavax B	a	7/7*	6/6	5/7	1/7		3.8
Aqu. HBsAg	a	4/6	3/7	0/7	0/6		180
Aqu. HBsAg	b	6/7	0/7	1/7	0/7		240
Aqu. HBsAg	c	1/7	0/7	0/7	0/7		>2000

* Number positive seroconversions per number vaccinated.

a 3 injections of 1/3 total dose a month apart.

b Injections administered every three days for 90 days in decreasing dosages according to a logarithmic progression.

c Injections of 1/90 total dose daily for 90 days.

effect on potency than did the vaccination regimen, and that pulsing with large doses of immunogen was more effective than continuous administration of small doses.

HBsAg release from DL:PLG microcapsules. The microcapsules employed in this study were designed to

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disintegrate within three weeks after hydration. It is evident from the release curve (Fig. 2) that they performed as designed, releasing approximately 17% of their total load in an initial pulse and approximately 7% of the remaining available HBsAg over the first three weeks.

Assessment of the potency of DL:PLG

microencapsulated HBsAg for immunizing ICR mice when used alone and in combination with Heptavax B vaccine.

The results (Table 6) indicate that the microencapsulated HBsAg had approximately the same immunogenicity as did the Heptavax B. Neither immunogens were sufficiently potent to effect with a singly injection seroconversion rates similar to those achieved after three injections of Heptavax B (Table 4). Only the immunogen

TABLE 6. Potencies of Heptavax B and microencapsulated HBsAg by single injection S.C. when administered alone and in combination to immunize ICR mice.

Var. Dose	ng Const.	ng Variable Dose				Var. Dose Tot.	
Dose							
Immunogen Dose	mHBsAg	2500	250	25	2.5	.25	ED ₅₀ ng
ED ₅₀	ng						

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1	Heptavax B	0	13/14*	8/14	4/14	0/13	130
2	130						
3	Heptavax B	0.16		11/13	4/14	1/14	1.7
4	1.8						
5	Heptavax B	1.6		10/13	1/14	0/13	100
6	100						
7	Heptavax B	16		3/14	1/14	1/14	>470
8	>490						
9	Heptavax B	160		3/12	2/11	1/12	>370
10	>530						
11	Heptavax B	1600		7/7	7/7	7/7	<0.8
12	1600						
13	Mic. HBsAg	0	3/6	6/15	1/13	2/10	2/14 220
14	220						

15

16

17 * Number positive seroconversions per number vaccinated.

18 combination of Heptavax B with 0.16 ng mHGsAg provided
 19 this level of seroconversion. At the ED₅₀ endpoint, the
 20 0.16 ng dose of mHGsAg is approximately 10% of the
 21 total dose. Similarly, a small amount of Heptavax B
 22 appeared to enhance the immunogenicity of the
 23 microencapsulated immunogen, although the combination
 24 was clearly less immunogenic when the two formulations
 25 were present at equivalent concentrations.

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TABLE 7. ANTIBODY SECRETING CELL RESPONSES TO CFA/II VACCINE BY ELISPOT ASSAY
AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS
0, 7, 14, AND 28 (E. COLI CVD 15001)

	vaccinee	IgA				IgG				IgM			
		pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7
6	15001-1	0	0	0	0	0	0	0	0	0	0	0	0
7	15001-2	0	0	0	0	0	0	0	0	0	0	0	0
8	15001-3'	0	22	0	0	10	0	0	0	0	0	0	0
9	15001-4	0	6	0	0	16	0	0	0	0	0	0	0
10	15001-6'	0	0	0	0	0	0	0	0	0	0	0	0
11	15001-7	0	0	0	0	0	0	0	0	0	0	0	0
12	15001-8	0	0	0	0	2	0	0	0	0	0	0	0
13	15001-9	0	520	4	0	16	0	256	0	0	52	0	8
14	15001-10	0	0	0	0	50	0	0	0	0	0	0	0
15	15001-11	0	180	32	0	30	0	56	0	0	0	0	0

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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.

TABLE 8. ANTIBODY SECRETING CELL RESPONSES TO CS1 BY ELISPOT ASSAY AFTER VACCINATION
WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28
(E. COLI CVD 15001)

Vaccinee	IgA				IgG				IgM			
	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7
15001-1	0	0	0	0	0	0	0	0	0	0	0	0
15001-2	0	0	0	0	0	0	0	0	0	0	0	0
15001-3 ¹	0	0	0	0	0	0	0	0	0	0	0	0
15001-4	0	0	0	0	0	0	0	0	0	0	0	0
15001-6 ²	0	0	0	0	0	0	0	0	0	0	0	0
15001-7	0	0	0	0	0	0	0	0	0	0	0	0
15001-8	0	0	0	0	0	0	0	0	0	0	0	0
15001-9	0	128	0	0	12	56	118	0	2	0	0	0
15001-10	0	6	0	0	0	0	0	0	0	0	0	0
15001-11	0	140	0	0	0	0	34	0	0	0	2	0

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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.

TABLE 9. ANTIBODY SECRETING CELL RESPONSES TO C83 BY ELISPOT ASSAY AFTER VACCINATION
WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28
(E. COLI CVD 15001)

	Vaccinee	IgA				IgG				IgM			
		pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7
1													
2													
3													
4													
5													
6	15001-1	0	0	0	0	0	0	0	0	0	0	0	0
7	15001-2	0	0	0	0	0	0	0	0	0	0	0	0
8	15001-3'	0	0	0	0	26	0	0	0	0	0	0	0
9	15001-4	0	0	0	0	98	0	0	0	0	30	0	0
10	15001-6'	2	0	0	0	0	0	0	0	0	0	0	0
11	15001-7	0	0	0	0	0	0	0	0	0	0	0	0
12	15001-8	0	0	0	0	0	0	0	0	0	0	0	0
13	15001-9	0	580	4	0	6	0	336	0	0	12	0	4
14	15001-10	0	0	0	0	88	0	0	0	0	0	0	0
15	15001-11	0	162	32	0	8	0	12	2	0	0	0	0

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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.

TABLE 10. ANTIBODY SECRETING CELL RESPONSES TO C83 PEPTIDE 795 BY ELISPOT ASSAY AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28

(E. COLI CVD 15001)

[illegible]

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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.

TABLE 11. ANTIBODY SECRETING CELL RESPONSES TO C83 PEPTIDE 792 BY ELISPOT ASSAY AFTER VACCINATION
WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28

(E. COLI CVD 15001)

4	Vaccinee	IgA				IgG				IgM				145			
		pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7		+14	+21	+35
5																	
6	15001-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	15001-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	15001-3 ¹	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	15001-4	0	0	0	0	2	0	0	0	0	0	0	0	0	0	12	
10	15001-6 ²	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	15001-7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	
12	15001-8	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	15001-9	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	15001-10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	15001-11	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.

TABLE 12. JEJUNAL FLUID SECRETORY IGA RESPONSES (RECIPROCAL TITER) TO CFA/II BY ELISA AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28

(E. COLI CVD 15001)

Vaccinee	Pre	+8	+14	+28	+35
15001-1	<4	<4	<4	<4	<4
15001-2	<4	IS	IS	IS	IS
15001-3 ¹	4	<4	NS	4	<4
15001-4	4	<4	4	4	16 ⁺
15001-6 ¹	4	NS	NS	NS	8
15001-7	<4	<4	4	4	32 ⁺
15001-8	8	4	4	8	8
15001-9	16	≥256	≥256	≥256	256 ⁺
15001-10	<4	<4	<4	<4	8 ⁺
15001-11	16	32	64	64	32 ⁺

¹Received third dose of vaccine intragastrically.

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1 Received second, third, and fourth doses of vaccine intragastrically.

2 NS indicates no sample. IS indicates inadequate sample.

3 + indicates significant rise in titer.

TABLE 13. SERUM ANTIBODY RESPONSES (RECIPROCAL TITERS) TO CFA/II BY ELISA AFTER VACCINATION
WITH CFA/II VACCINE ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 21, AND 28
(E. COLI CVD 15001)

Vaccinee	IgG				IgA				IgM			
	Pre	+7	+28		Pre	+7	+28		Pre	+7	+28	
15001-1	400	100	100		50	50	50		50	50	50	
15001-2	6400	3200	6400		25	<25	<25		50	50	25	
15001-3 ¹	3200	6400	6400		100	200	50		100	50	50	
15001-4	400	200	400		100	400	100 ⁺		100	50	50	
15001-6 ²	1600	1600	1600		200	200	200		25	25	25	
15001-7	400	6400	3200 ⁺		400	200	200		25	25	50	
15001-8	3200	400	400		800	800	200		25	25	25	
15001-9	6400	12800	6400		800	3200	3200 ⁺		50	50	50	
15001-10	800	400	400		400	400	200		200	400	200	
15001-11	800	1600	1600		400	400	100		25	25	25	

¹Received third dose of vaccine intragastrically.

²Received second, third, and fourth doses of vaccine intragastrically.

+ indicates significant rise in titer.

TABLE 14. SERUM ANTIBODY RESPONSES (RECIPROCAL TITERS) TO CS1 BY ELISA AFTER VACCINATION
WITH CFA/II VACCINE ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 21, AND 28
(E. COLI CVD 15001)

Vaccinee	IgG				IgA				IgM			
	Pre	+7	+28		Pre	+7	+28		Pre	+7	+28	
15001-1	<25	<25	<25		25	<25	<25		<25	<25	<25	
15001-2	<25	<25	25		<25	<25	<25		<25	<25	<25	
15001-3 ¹	<25	<25	<25		<25	<25	<25		<25	<25	25	
15001-4	<25	<25	<25		25	<25	<25		25	25	<25	
15001-6 ²	<25	<25	<25		25	<25	<25		<25	<25	<25	
15001-7	<25	<25	<25		<25	25	<25		<25	<25	<25	
15001-8	<25	<25	<25		25	<25	25		<25	<25	<25	
15001-9	800	800	400		200	200	200		<25	<25	<25	
15001-10	<25	<25	<25		25	<25	<25		25	<25	25	
15001-11	200	3200	800 ⁺		100	200	200		<25	<25	<25	

¹Received third dose of vaccine intragastrically.

²Received second, third, and fourth doses of vaccine intragastrically.

+ indicates significant rise in titer.

TABLE 15. SERUM ANTIBODY RESPONSES (RECIPROCAL TITERS) TO CS3 BY ELISA AFTER VACCINATION
WITH CFA/II VACCINE ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 21, AND 29
(E. COLI CVD 15001)

Vaccinee	IgG				IgA				IgM			
	Pre	+7	+28		Pre	+7	+28		Pre	+7	+28	
15001-1	50	50	50		50	25	25		<25	25	<25	
15001-2	800	1600	800		<25	<25	<25		25	25	25	
15001-3 ¹	<25	25	50 ⁺		50	25	25		25	50	50	
15001-4	25	100	25 ⁺		50	50	50		<25	25	25	
15001-6 ²	200	200	200		200	50	100		25	50	100 ⁺	
15001-7	100	50	<25		100	50	25		50	<25	<25	
15001-8	<25	200	100 ⁺		25	50	25		<25	50	50 ⁺	
15001-9	100	800	800 ⁺		50	400	400 ⁺		25	50	25	
15001-10	200	100	100		50	25	50		25	25	100 ⁺	
15001-11	100	100	200		50	50	50		25	25	<25	

¹Received third dose of vaccine intragastrically.

²Received second, third, and fourth doses of vaccine intragastrically.

+ indicates significant rise in titer.

TABLE 16. CLINICAL AND BACTERIOLOGIC RESPONSES TO CHALLENGE WITH 5×10^7 CFU OF
ENTEROTOXIGENIC *E. COLI* STRAIN E24377A (O139:H28 LT'ST'CS'CS3') AMONG
VACCINEES AND CONTROL VOLUNTEERS (*E. COLI* CVD 15002)

Volunteer	Inoculation Period (hr:min)	Volume of Grade 2, 3 Sample (ml)	No. of Sheds Grade 2, 3	Time (min) (17)	Duration of Fecal Shedding (days)	Peak Shed Concentration (10^6 /g fec)
Vaccinees						
15001.1	18:20	1391	10	-	7	1×10^7
15001.2	41:30	817	3	-	6	1×10^8
15001.3	30:07	1231	9	1×10^2 3	5	3×10^7
15001.4	21:18	1012	7	1×10^2 3	7	1×10^7
15001.6	-	0	0	-	5	2×10^7
15001.7	18:48	6380	19	-	6	6×10^8
15001.8	21:19	9132	41	1×10^1 9	5	9×10^8
15001.9	-	0	0	-	5	3×10^7
15001.10	21:08	1608	14	-	7	3×10^8
15001.11	-	0	0	-	5	2×10^7
Mean	23:34	2019	10.7	-	5.8	3×10^8
Control Volunteers						
15002.1	15:51	1701	0	-	4	3×10^8
15002.5	24:01	817	0	-	8	3×10^8
15002.6	21:40	179	7	-	4	2×10^8
15002.8	13:58	1753	6	1×10^1 4	7	3×10^7
15002.9	22:12	1518	11	-	4	3×10^8
15002.11	27:14	1253	7	-	7	4×10^8
15002.12	20:31	1738	13	-	5	3×10^8
15002.13	21:58	740	5	-	6	5×10^8
15002.16	48:07	1004	7	-	6	1×10^8
15002.21	20:11	1448	18	-	5	5×10^8
Mean	23:38	1464	8.6	-	5.8	3.8×10^8

TABLE 17. ANTIBODY SECRETING CELL RESPONSES TO CFA/II, CS1, AND CS3 BY ELISPOT AFTER CHALLENGE WITH ENTEROTOXIGENIC E. COLI STRAIN E24377A AMONG VACCINEES AND CONTROL VOLUNTEERS
(E. COLI CVD 15002)

Volunteer	CFA/II						CS1						CS3					
	Pre ¹	1:7	Pre ²	1:7	Pre ³	1:7	Pre ¹	1:7	Pre ²	1:7	Pre ³	1:7	Pre ¹	1:7	Pre ²	1:7	Pre ³	1:7
Vaccinees																		
15001 1	0	40	0	0	0	12	21	0	0	0	0	0	0	0	0	0	0	0
15001 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001 3 ¹	12	202	40	34	0	29	0	0	0	0	0	0	0	0	0	0	0	0
15001 4	14	218	0	200	0	5	0	0	0	0	0	0	0	0	0	0	0	0
15001 5 ¹	0	24	16	0	0	34	0	0	0	0	0	0	0	0	0	0	0	0
15001 6	0	12	0	0	24	28	0	0	0	0	0	0	0	0	0	0	0	0
15001 7	0	18	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
15001 8	24	180	208	36	0	2	0	37	11	10	0	45	0	0	0	0	0	0
15001 9 ¹	740	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001 10	34	340	0	217	0	88	0	0	0	0	0	0	0	0	0	0	0	0
15001 11 ¹	0	38	0	2	0	2	17	0	0	0	0	0	0	0	0	0	0	0
Controls																		
15002 1	0	200	0	0	0	24	0	0	0	0	0	0	0	0	0	0	0	0
15002 2	0	14	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0
15002 3	0	14	0	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0
15002 4	0	14	0	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0
15002 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002 21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

¹Received third dose of vaccine on day 14.
²Received second, third and fourth doses of vaccine on days 14, 21, 28 and 35.
³Pre-vaccination blood sample taken on day 0.
⁴Vaccinees who did not become ill.

TABLE 18. ANTIBODY SECRETING CELL RESPONSES TO CS3 PEPTIDES 792 AND 795 BY ELISPOT AFTER CHALLENGE WITH ENTEROTOXIGENIC E. COLI STRAIN E24377A AMONG VACCINEES AND CONTROL VOLUNTEERS
(E. COLI CVD 15002)

Volunteer	CS3 PEPTIDE 792						CS3 PEPTIDE 795					
	Pre ¹	Day 7	Day 14	Day 21	Day 28	Day 35	Pre ¹	Day 7	Day 14	Day 21	Day 28	Day 35
Vaccinees												
15001.1	0	0	0	0	0	0	0	0	0	0	0	0
15001.2	0	0	0	0	0	0	0	0	0	0	0	0
15001.3 ²	0	0	0	0	0	0	0	0	0	0	0	0
15001.4	0	0	0	0	0	0	0	0	0	0	0	0
15001.5 ^{2,4}	0	0	0	0	0	0	0	0	0	0	0	0
15001.6	0	0	0	0	0	0	0	0	0	0	0	0
15001.7	0	0	0	0	0	0	0	0	0	0	0	0
15001.8	22	7	0	0	0	0	0	0	0	0	0	0
15001.9 ⁴	0	0	0	0	0	0	0	0	0	0	0	0
15001.10	7	0	0	0	0	0	7	0	0	0	0	0
15001.11 ⁴	0	0	0	0	0	0	0	0	0	0	0	0
Controls												
15002.1	0	0	0	0	0	0	0	0	0	0	0	0
15002.5	0	0	0	0	0	0	0	0	0	0	0	0
15002.6	0	0	0	0	0	0	0	0	0	0	0	0
15002.8	0	0	0	0	0	0	0	0	0	0	0	0
15002.9	0	0	0	0	0	0	0	0	0	0	0	0
15002.11	0	0	0	0	0	0	0	0	0	0	0	0
15002.12	0	0	0	0	0	0	0	0	0	0	0	0
15002.13	0	0	0	0	0	0	0	0	0	0	0	0
15002.16	7	10	0	0	0	0	7	0	0	0	0	0
15002.21	0	0	0	0	0	0	0	0	0	0	0	0

¹Prechallenge cell count of vaccinee biologically

²Pre and second, third, and fourth doses of vaccine biologically

³Pre indicates before challenge, for vaccinees, Pre is day 37 after first dose of vaccine

⁴Vaccinees who did not become ill

TABLE 19. ANTIBODY SECRETING CELL RESPONSES TO 0139 LIPOPOLYSACCHARIDE (LPS) AND HEAT LABILE ENTEROTOXIN (LT) BY ELISPOT AFTER CHALLENGE WITH ENTEROTOXIGENIC E. COLI STRAIN E24377A AMONG VACCINEES AND CONTROL VOLUNTEERS (E. COLI CVD 15002)

Volunteer	0139 LPS										LT									
	Day 1		Day 2		Day 3		Day 4		Day 5		Day 1		Day 2		Day 3		Day 4		Day 5	
	Pos ¹	Pos ²	Pos ¹	Pos ²	Pos ¹	Pos ²	Pos ¹	Pos ²	Pos ¹	Pos ²	Pos ¹	Pos ²	Pos ¹	Pos ²	Pos ¹	Pos ²	Pos ¹	Pos ²	Pos ¹	Pos ²
Vaccinees																				
15001.1	10	116	0	112	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.3 ³	0	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.4	0	500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.5 ^{2,4}	0	190	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.7	0	314	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.8	24	240	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.9 ⁴	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.10	2	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.11 ⁴	0	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Controls																				
15002.1	0	100	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.5	0	400	0	170	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.6	0	117	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.8	0	317	0	140	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.9	0	440	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.11	0	140	0	170	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.12	0	117	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.13	0	370	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.16	0	714	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.21	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

¹ Positives at day 1 of vaccinee group only.
² Positives at day 2 of vaccinee group only.
³ Positives at day 3 of vaccinee group only.
⁴ Positives at day 4 of vaccinee group only.

1 TABLE 20. IMMUNE RESPONSES AS MEASURED BY ANTIBODY SECRETING CELLS (ASC) AND BY JEJUNAL
2 FLUID SECRETORY IGA AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE
3 MICROSPHERES ON DAYS 0, 7, 14, AND 28

Immunologic Assay	Number of Responders ¹	Geometric mean peak number of spots per 10 ⁶ PBMC (ASC) or reciprocal antibody titer (sIgA)
ASC IgA anti-CFA/II	5/10	44
ASC IgA anti-CS1	3/10	48
ASC IgA anti-CS3	5/10	116
Jejunal fluid sIgA anti-CFA/II	5/10	42

¹Responses that had occurred by day 35 after the first dose of vaccine, i.e., day 7 after the fourth dose

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TABLE 21. IMMUNE RESPONSES AFTER WILD-TYPE ETEC CHALLENGE AS MEASURED BY
ANTIBODY SECRETING CELLS (ASC) AND BY JEJUNAL FLUID SECRETORY IGA IN
UNIMMUNIZED CONTROL VOLUNTEERS

Immunologic Assay	Number of Responders ¹	Geometric mean peak number of spots per 10 ⁶ PBMC (ASC) or reciprocal antibody titer (sIgA)
ASC IgA anti-CFA/II	9/10	88
ASC IgA anti-CS1	4/10	58
ASC IgA anti-CS3	9/10	161
Jejunal fluid sIgA anti-CFA/II	6/9	72

¹Measured day 7 after challenge

TABLE 22. PRE-CHALLENGE IMMUNITY AND CLINICAL AND BACTERIOLOGIC RESPONSE
TO CHALLENGE WITH 5×10^8 CFU OF ENTEROTOXIGENIC E. COLI STRAIN E24377A
(0139:H28 LT⁺ST⁺CS1⁺CS3⁺) AMONG VACCINEES AND CONTROL VOLUNTEERS

	Vaccinees	Controls
Number with > 4 IgA anti-colonization factor ASC ¹ per 10^6 PBMC on the day of challenge ²	8/10	4/10
Geometric mean number of IgA anti-colonization factor ASC per 10^6 PBMC on the day of challenge ³	25	14
Attack Rate for Diarrhea	7/10	10/10
Volume of Diarrhea	2819 ml	1464 ml
Peak Stool Excretion of Challenge Organism	3×10^8 cfu	4×10^8 cfu

¹Including anti-CFA/II, anti-CS1, and/or anti-CS3

²Day 57 after the first dose of vaccine

³Among those with > 4 IgA ASC before challenge (n=8 for vaccinees and n=4 for controls)

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DISCUSSION

The potential advantage of microcapsules lies in their ability to be programmed during fabrication into forms that have quite different release profiles, including slow and steady release, multiple bursts of antigen over a period of time, or combinations of release forms. Sieving allows choice of microcapsule size, and the ability of DL-PLG to sequester antigen from the host's immune system until release occurs enhances control over exposure of the recipient's immune system to antigen over a sustained period of time. These characteristics provided the impetus for these studies as they indicate potential for achieving the effects of a multiple injection regimen by controlling release in vivo after a single injection.

The results of these studies are important for gaining an understanding of the fundamental differences between the manner in which alum and microcapsules interact with the immune system. The antigen release studies showed that alum firmly bound the antigen on its surface, whereas the microcapsules sequestered the antigen load within the interstices of an immunologically inert polymer. Release of antigen from microcapsules was spontaneous and gradual while antigen release from alum was probably enzymatically mediated within host macrophages. Alum thus performed at least two useful functions as an adjuvant: by bearing its entire load of antigen upon its surface, it provided a large single exposure of antigen to the host; and, by being

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1 readily phagocytized by host macrophages, it served as a means
2 of targeting the antigen to the immune system.

3 In order for microcapsules to be efficacious as a vaccine
4 delivery system, a means of incorporating the two properties
5 common to alum adjuvant must be devised. These properties, which
6 were discussed above, are targeting antigen to the immune
7 system and delivering the antigen load in a single concentrated
8 pulse at its target. A gradual, sustained release of free
9 antigen, as was achieved with the 100 micron microcapsules used
10 in these studies, could be expected to elicit an immune response
11 similar to that seen with either regimen b or regimen c (Table
12 5), where multiple injections of small doses were employed. In
13 fact, as shown in Table 3, the microencapsulated immunogen
14 elicited a response similar to that achieved with regimen b.
15 This is probably due to the fact that the microcapsules release
16 approximately 10% of their antigenic load immediately after
17 injection.

18 Microcapsules with extended release patterns tend to be
19 large (>10 microns in diameter) and thus fail to be readily
20 phagocytized. In order for the larger microcapsules with
21 prolonged antigen release characteristics to be efficacious, the
22 antigen eventually released from those microcapsules would have
23 be in a form which targeted and concentrated it within the
24 recipient's immune system. This might be effectively achieved by
25 microencapsulation of antigen coated alum or by

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1 microencapsulating clusters of smaller (<10 microns)
2 microcapsules.

3 Microcapsules under 10 microns in diameter tend to be
4 readily phagocytized and also tend to under go rapid spontaneous
5 degradation due to their high surface to volume ratio. These
6 smaller microcapsules would be well suited for eliciting a
7 primary response if their pulse of antigen release could be
8 programmed to occur after phagocytosis.

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1 We Claim:

2 1. An immunostimulating composition comprising
3 encapsulating- microspheres, which may contain a
4 pharmaceutically-acceptable adjuvant, wherein said microspheres
5 having a diameter between 1 nanogram (ng) to 10 microns (um) are
6 comprised of (a) a biodegradable-biocompatible poly (DL-lactide-
7 co-glycolide) as the bulk matrix, wherein the relative ratio
8 between the amount of lactide and glycolide components are
9 within the range of 40:60 to 0:100 and (b) an immunogenic
10 substance comprising Colony Factor Antigen (CFA/II), hepatitis B
11 surface antigen (HBsAg), or a physiologically similar antigen
12 that serves to elicit the production of antibodies in animal
13 subjects.

14 2. An immunostimulating composition according to Claim 1
15 wherein the amount of said immunogenic substance is within the
16 range of 0.1 to 1.5% based on the volume of said bulk matrix.

17 3. An immunostimulating composition according to Claim 2
18 wherein the relative ratio between the lactide and glycolide
19 component is within the range of 48:52 to 58:42.

20 4. An immunostimulating composition according to Claim 2
21 wherein the size of more than 50% of said microspheres is
22 between 5 to 10 um in diameter by volume.

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1 5. An immunostimulating composition according to Claim 1
2 wherein the immunogenic substance is the synthetic peptide
3 representing the peptide fragment beginning with the amino acid
4 residue 63 through 78 of Pilus Protein CS3, said residue having
5 the amino acid sequence, 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-
6 His-Glu-Thr-Asn-Asn-Ser-Ala).

7 6. A vaccine comprising an immunostimulating composition
8 of Claim 4 and a sterile, pharmaceutically-acceptable carrier
9 therefor.

10 7. A vaccine comprising an immunostimulating composition
11 of Claim 6 wherein said immunogenic substance is Colony Factor
12 Antigen (CFA/II).

13 8. A vaccine comprising an immunostimulating composition
14 of Claim 6 wherein said immunogenic substance is hepatitis B
15 surface antigen (HBsAg).

16 9. A method for the vaccination against bacterial
17 infection comprising administering to a human, an
18 antibactericidally effective amount of a composition of Claim 6.

19 10. A method according to Claim 8 wherein the bacterial
20 infection is caused by a bacteria selected from the group
21 consisting essentially of Salmonella typhi, Shigella Sonnei,

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1 Shigella Flexneri, Shigella dysenteriae, Shigella boydii,
2 Escheria coli, Vibrio cholera, versinia, staphylococcus,
3 clostridium, and campylobacter.

4 11. A method for the vaccination against viral infection
5 comprising administering to a human an antivirally effective
6 amount of a composition of Claim 8.

7 12. A diagnostic assay for bacterial infections comprising
8 a composition of Claim 4.

9 13. A method of preparing an immunotherapeutic agent
10 against infections caused by a bacteria comprising the step of
11 immunizing a plasma donor with a vaccine according to Claim 7
12 such that a hyperimmune globulin is produced which contains
13 antibodies directed against the bacteria.

14 14. A method preparing an immunotherapeutic agent against
15 infections caused by a virus comprising the step of immunizing a
16 plasma donor with a vaccine according to Claim 8 such that
17 hyperimmune globulin is produced which contains antibodies
18 directed against the hepatitis B virus.

19 15. An immunotherapy method comprising the step of
20 administering to a subject an immunostimulatory amount of
21 hyperimmune globulin prepared according to Claim 13.

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1 16. An immunotherapy method comprising the step of
2 administering to a subject an immunostimulatory amount of
3 hyperimmune globulin prepared according to Claim 14.

4 17. A method for the protection against infection of a
5 subject by enteropathogenic organisms or hepatitis B virus
6 comprising administering to said subject an immunogenic amount
7 of an immunostimulating composition of Claim 3.

8 18. A method according to Claim 17 wherein the
9 immunostimulating composition is administered orally.

10 19. A method according to Claim 17 wherein the
11 immunostimulating composition is administered parenterally.

12 20. A method according to Claim 17, wherein the
13 immunostimulating composition is administered in four separate
14 doses on day 0, day 7, day 14, and day 28.

15 21. A method according to Claim 17 wherein the immunogenic
16 substance is the synthetic peptide representing the peptide
17 fragment beginning with the amino acid residue 63 through 78 of
18 Pilus Protein CS3 said residue having the amino acid sequence
19 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-His-Glu-Thr-Asn-Asn-Ser-
20 Ala).

Figure 1

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Particle Size Distribution

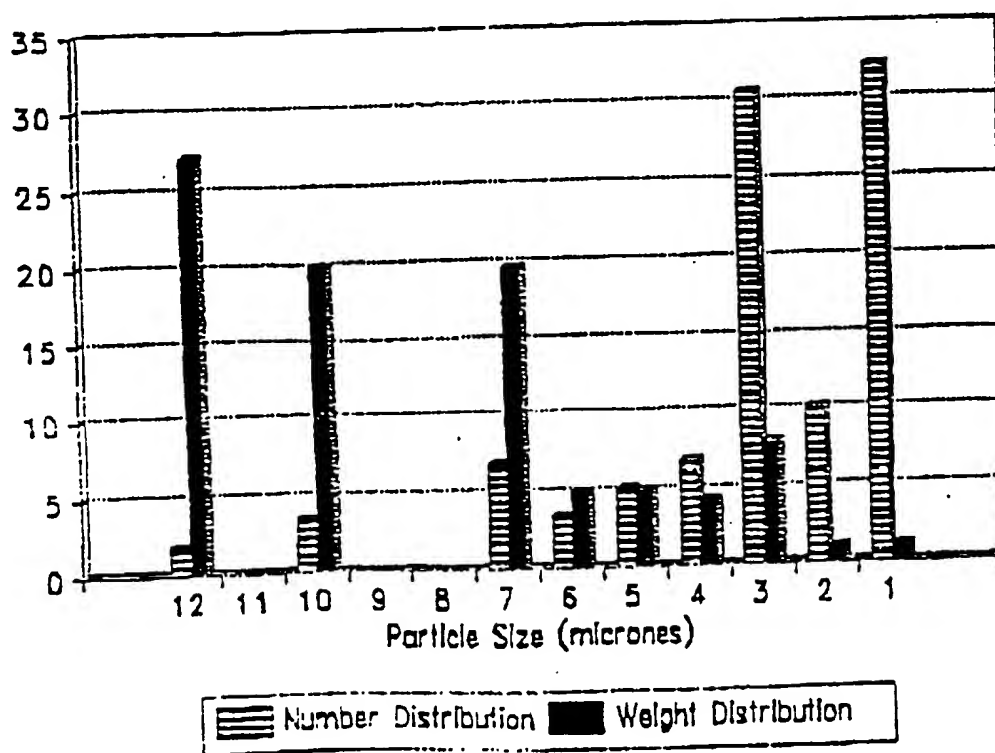


Figure 2

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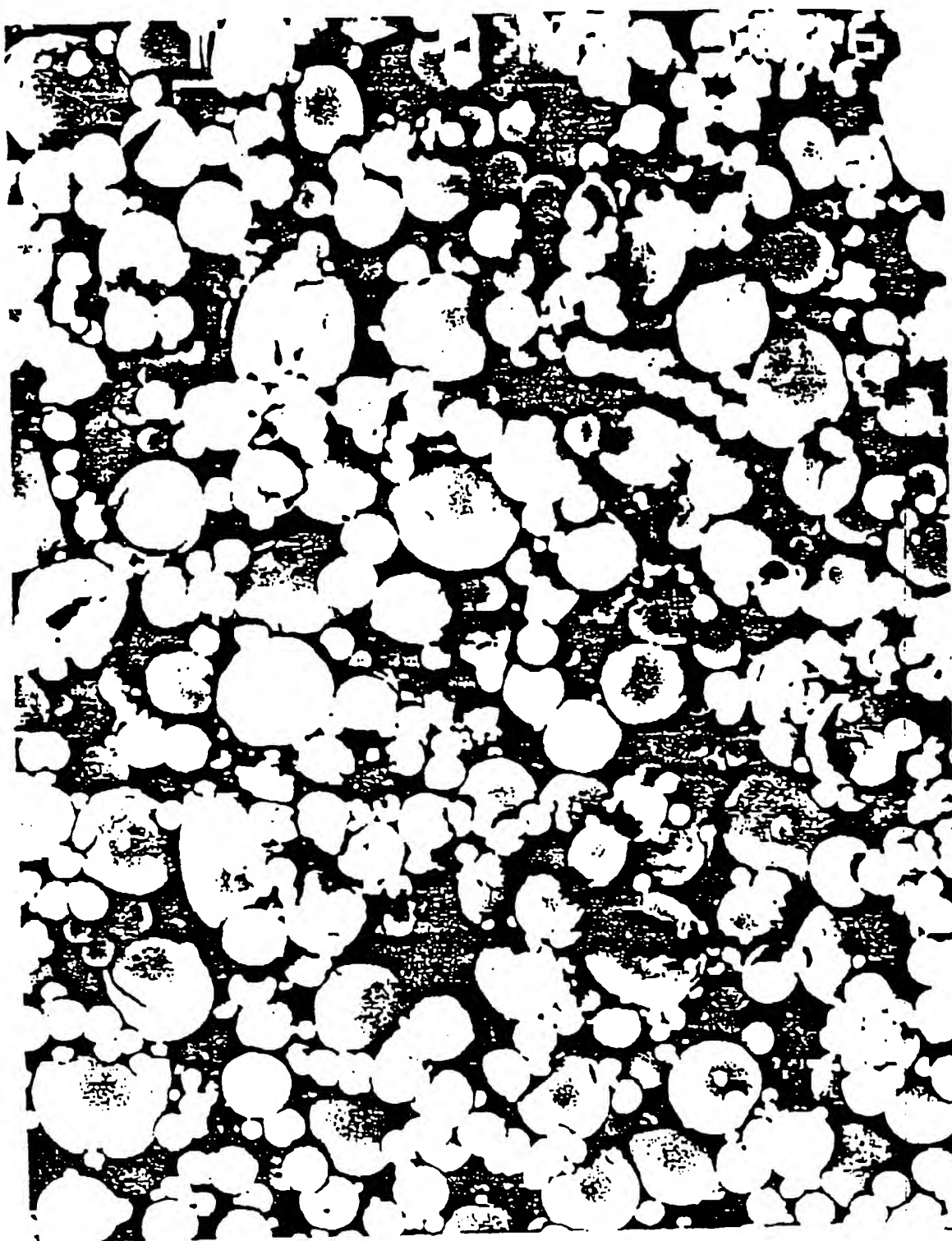


Fig. 3(a)
Spleen

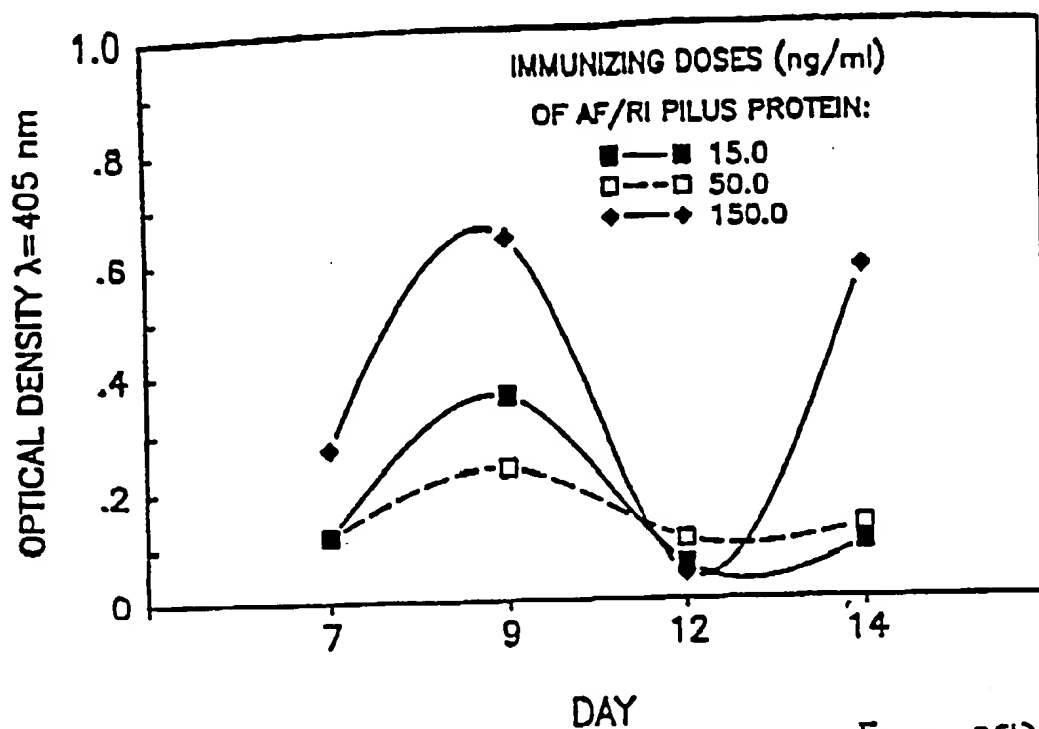
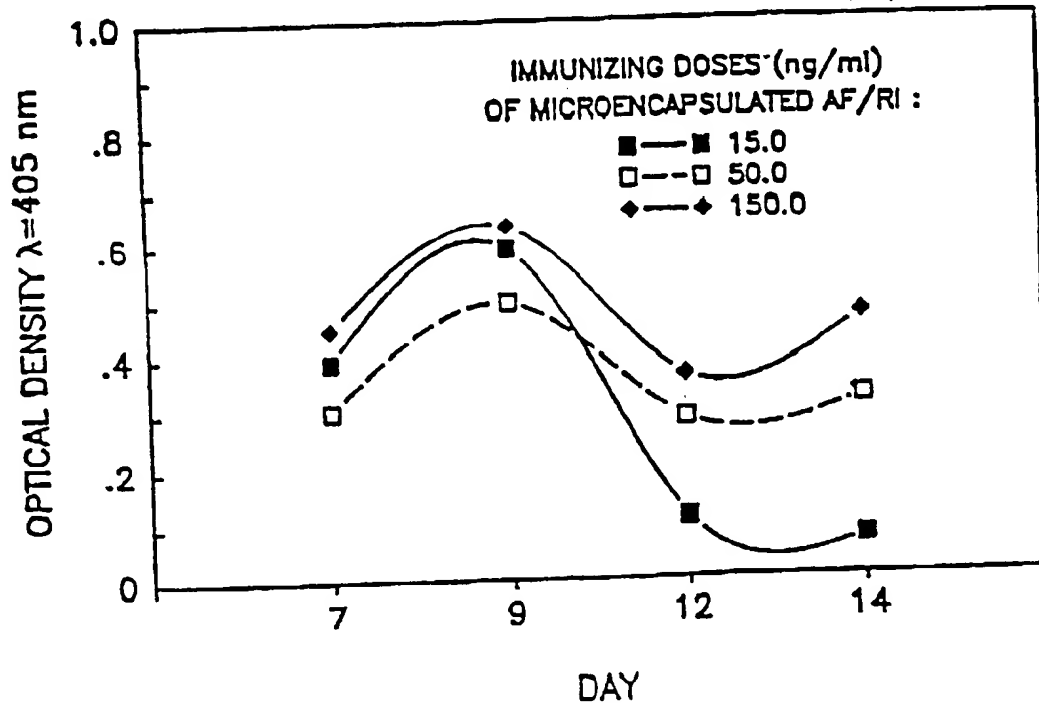


Figure 3(b)



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Figure 4(a)

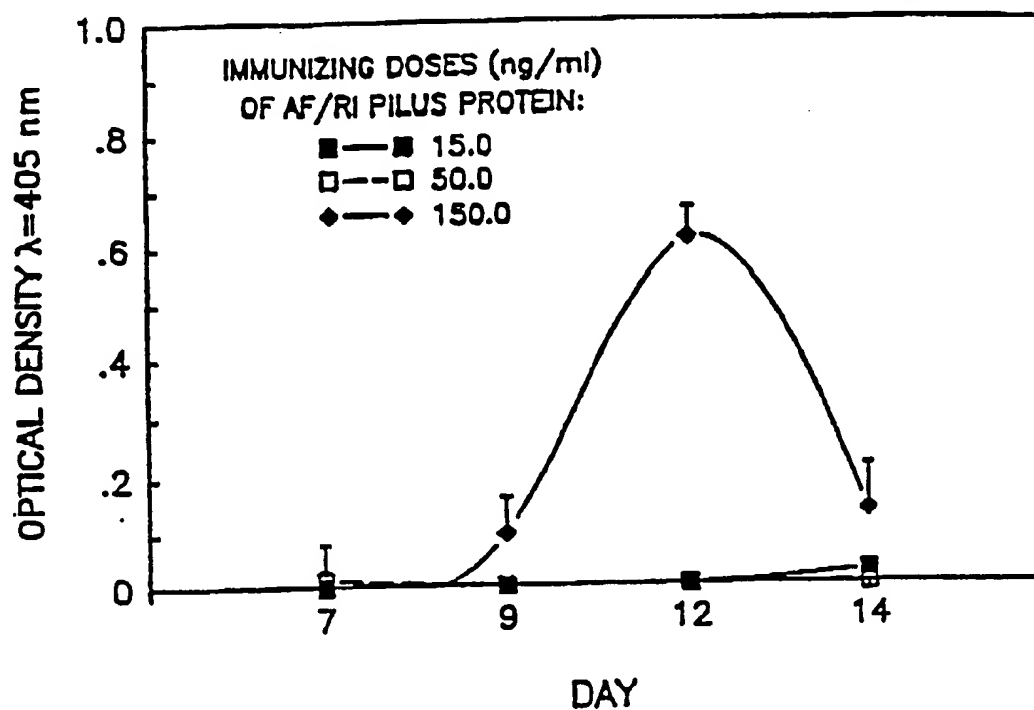
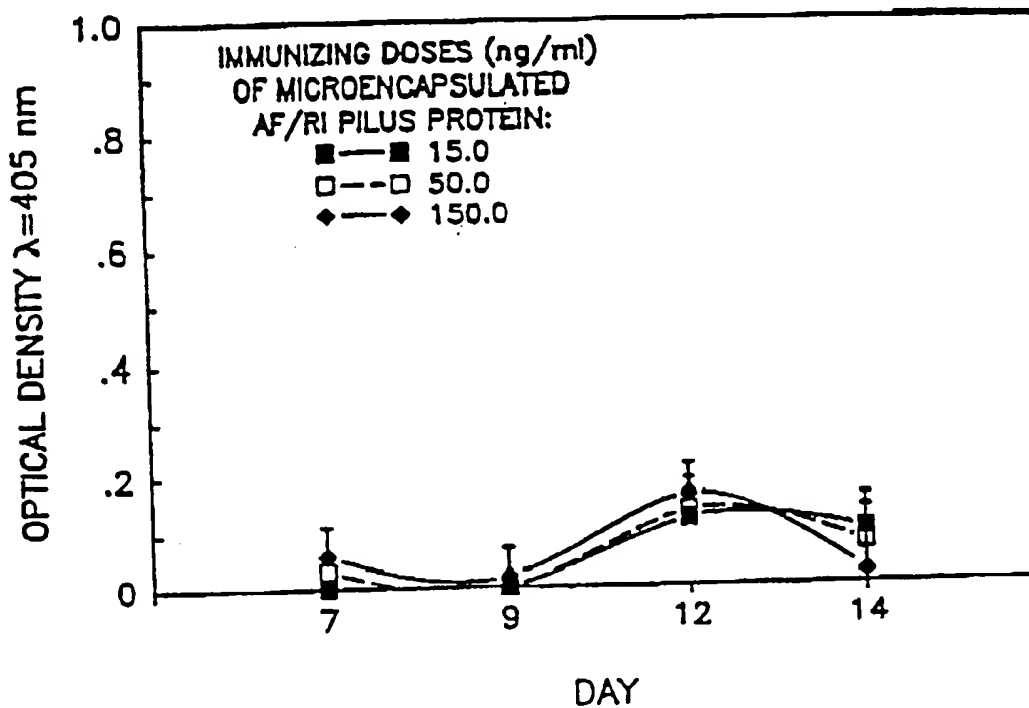


Figure 4(b)



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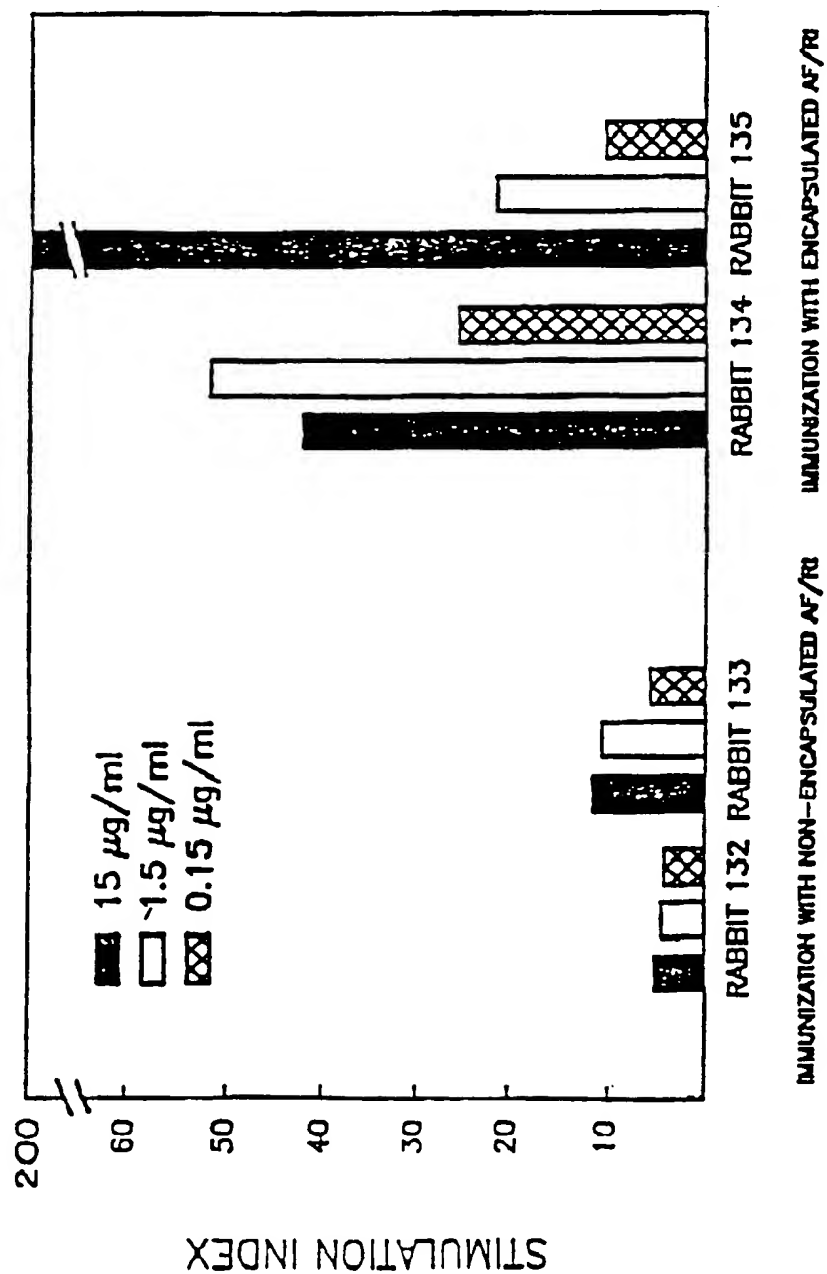
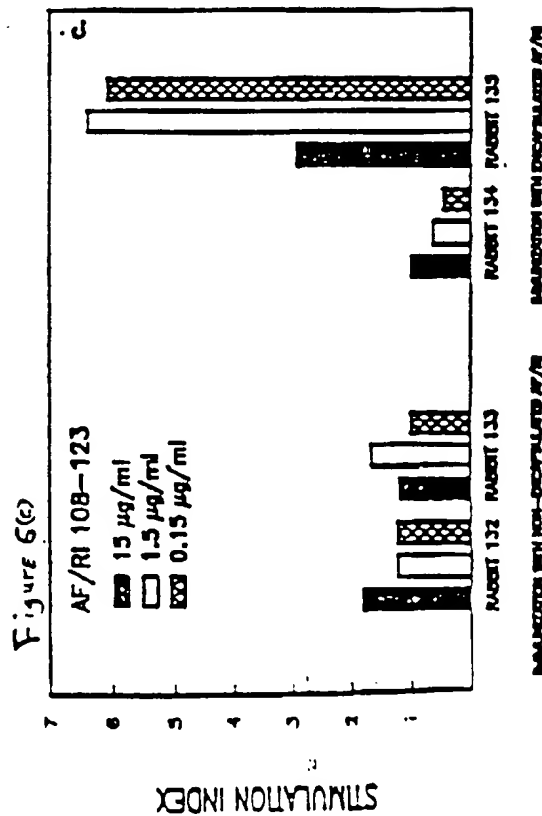
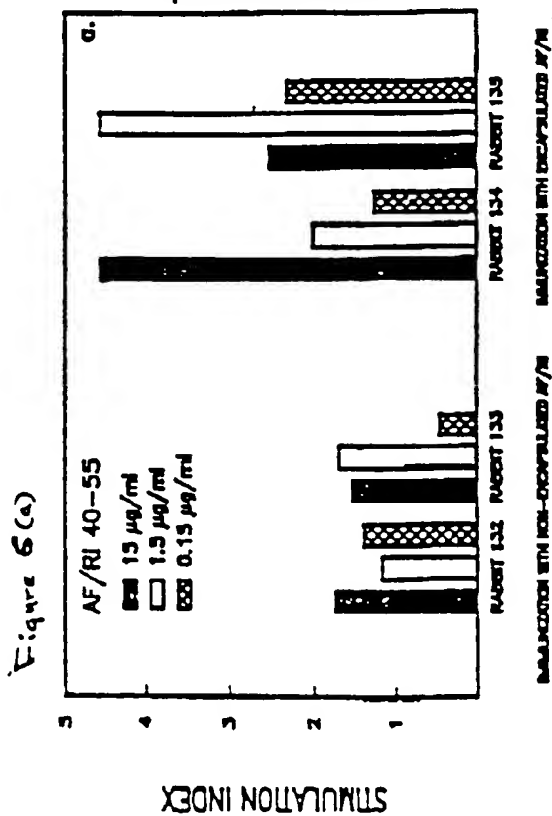
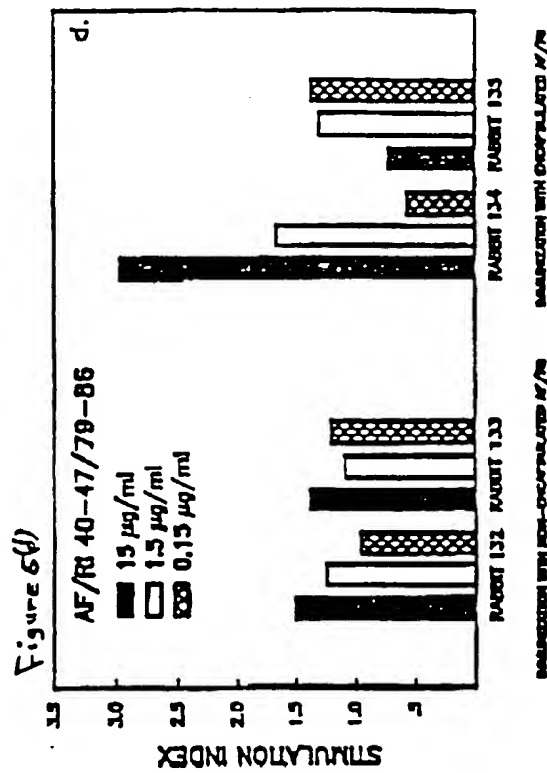
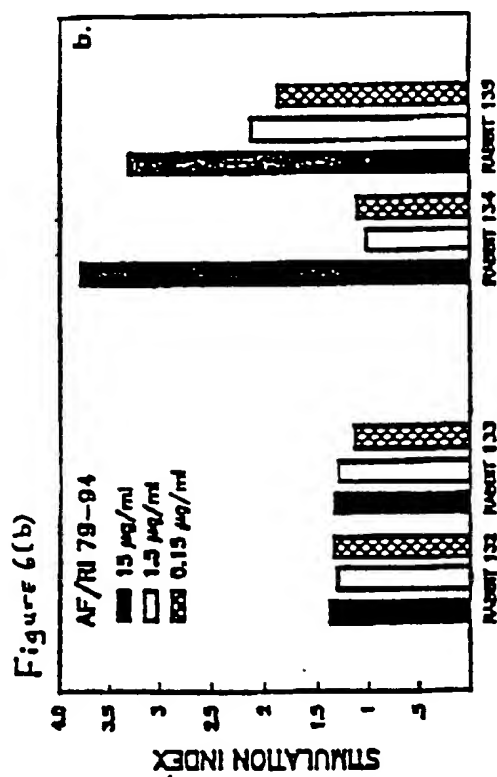


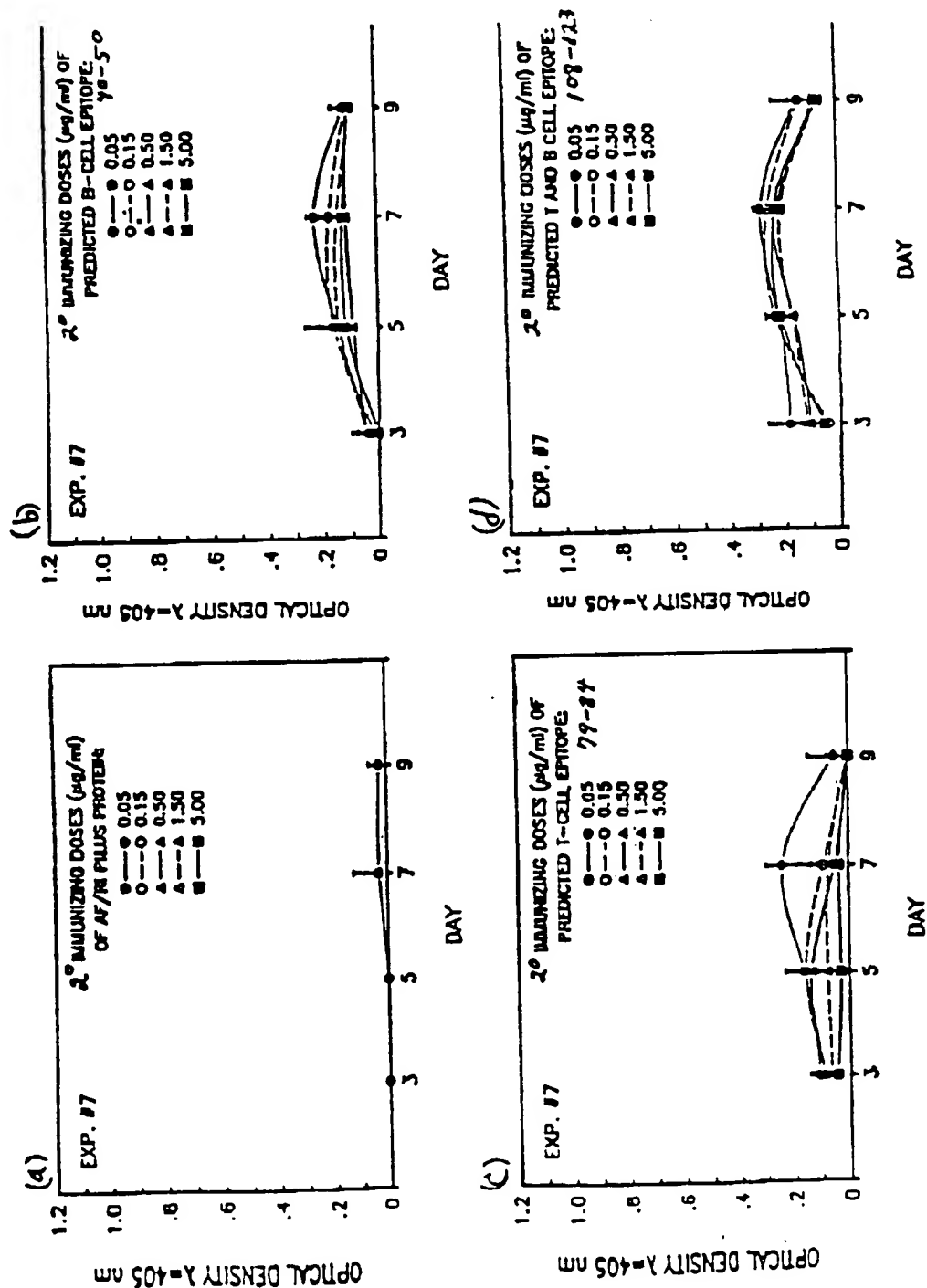
Figure 5

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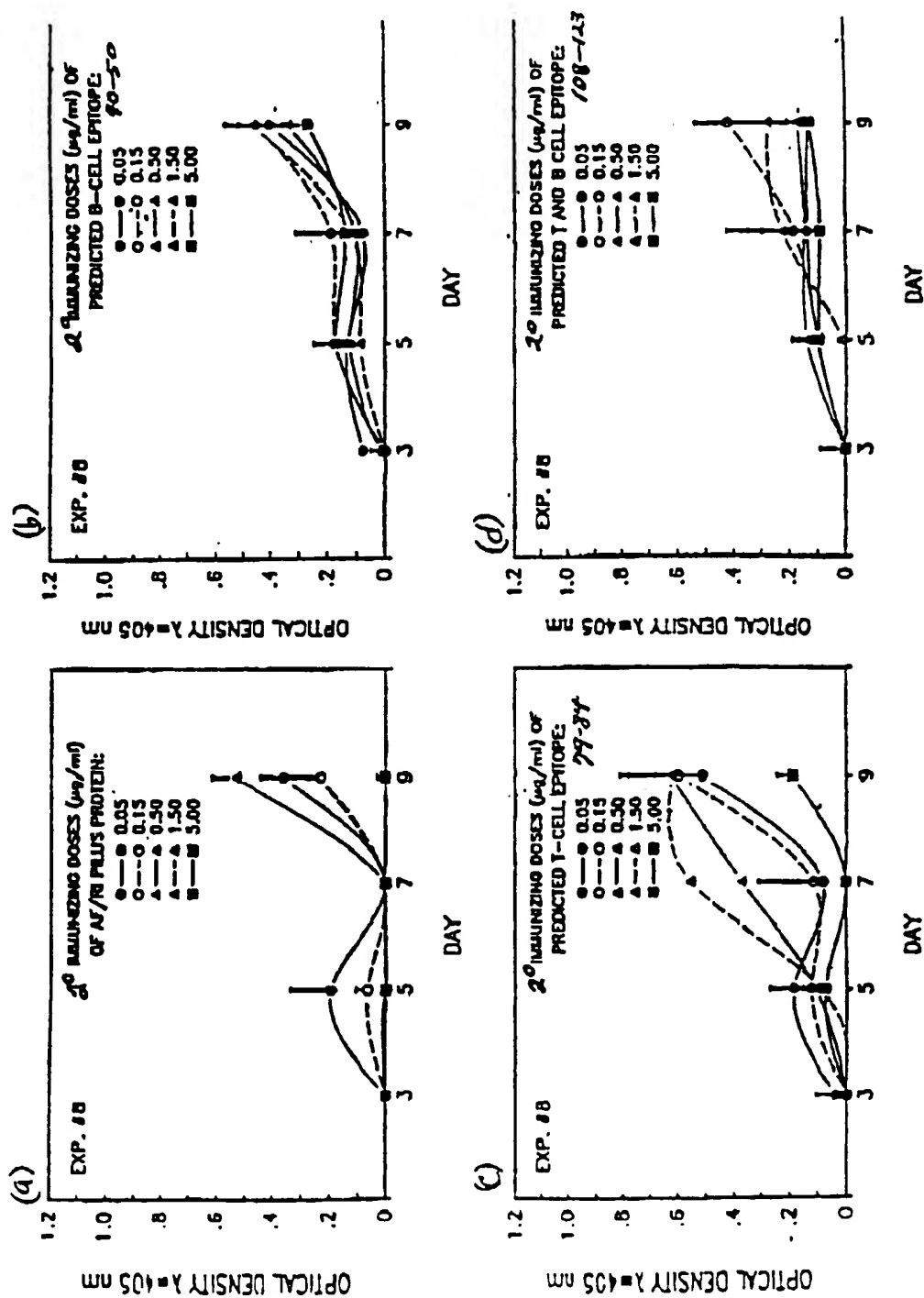
7 / 39

Fig. 7
Peyer's patch



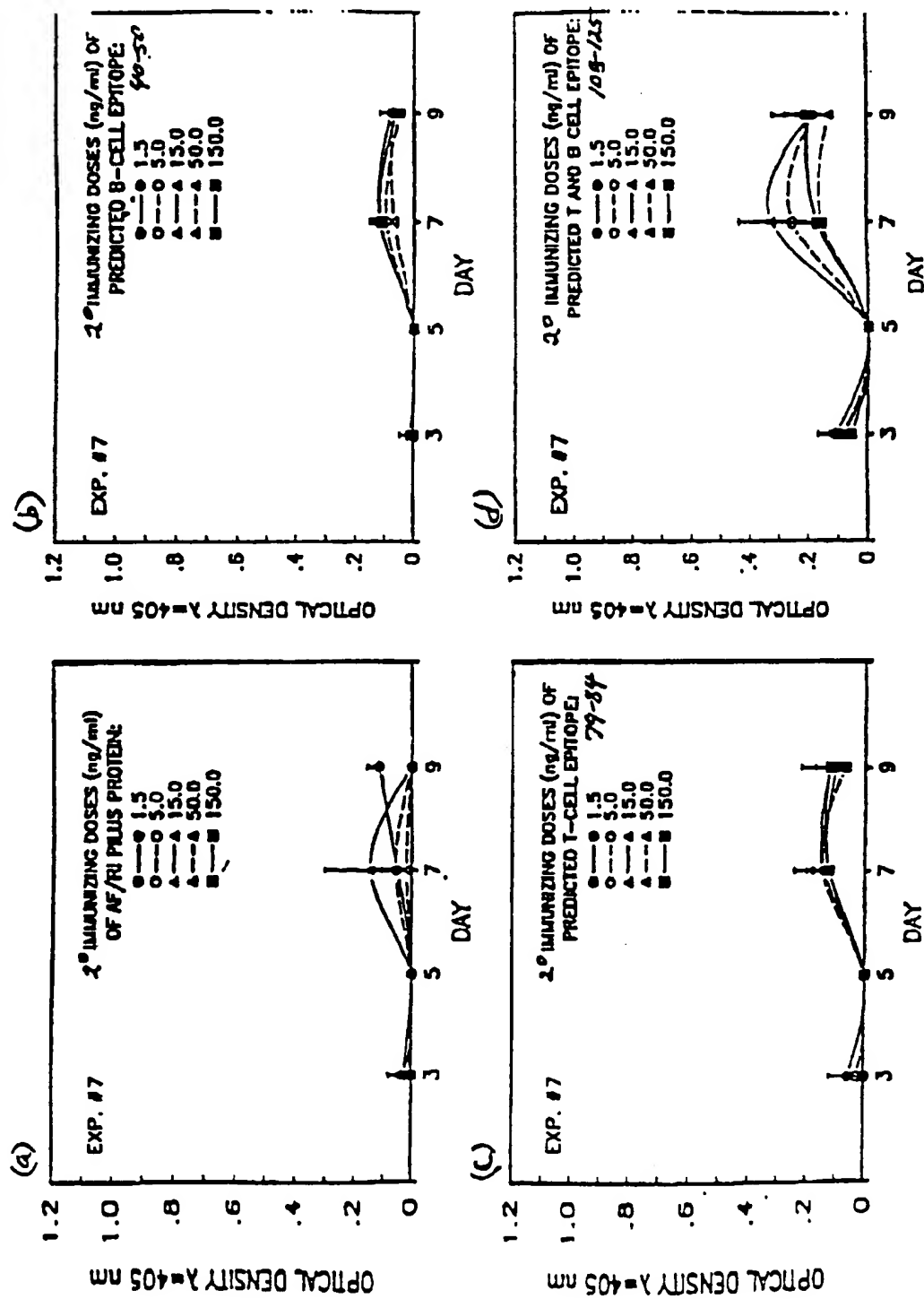
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Fig. 8



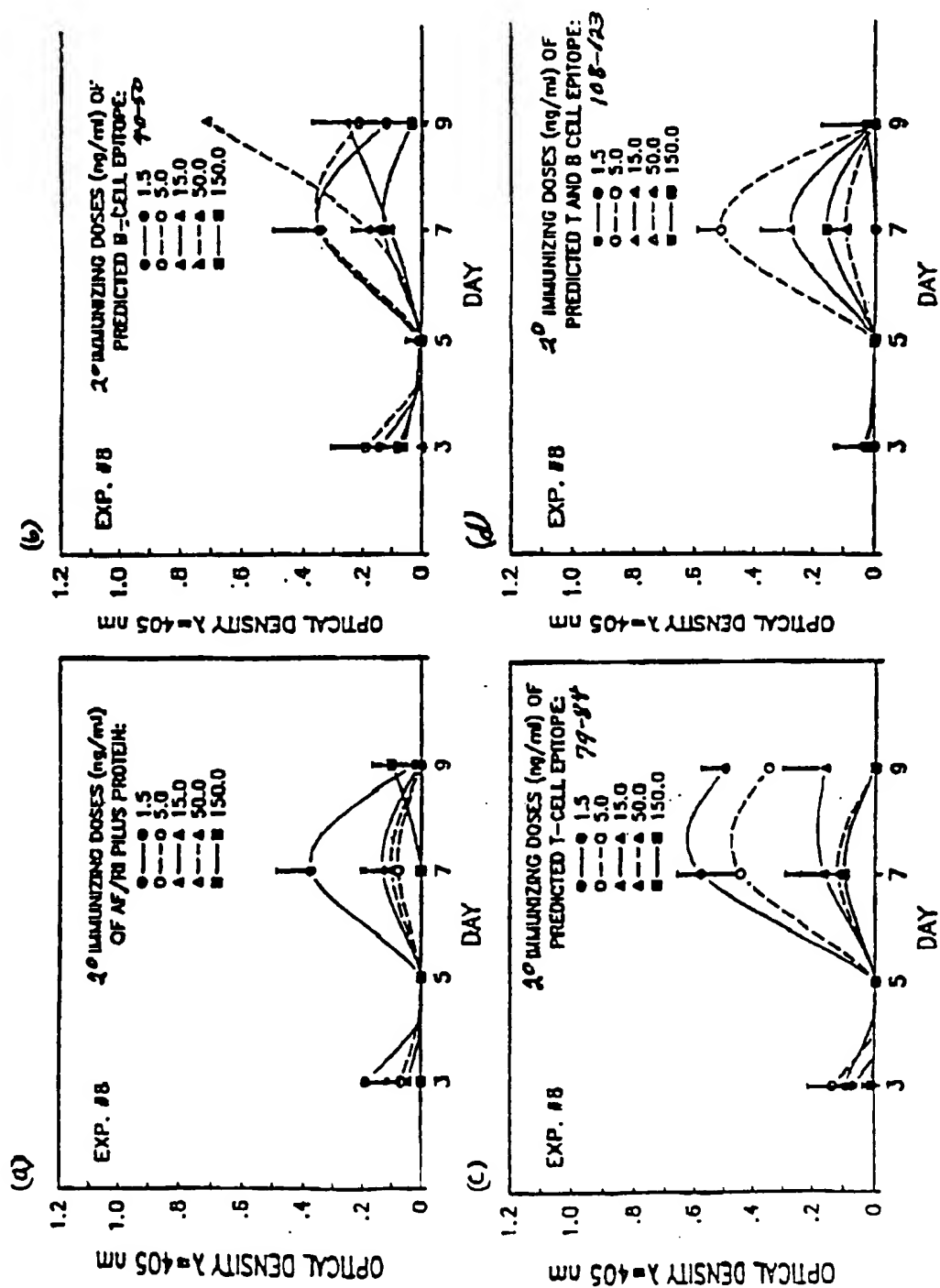
9 / 39

Fig. 9



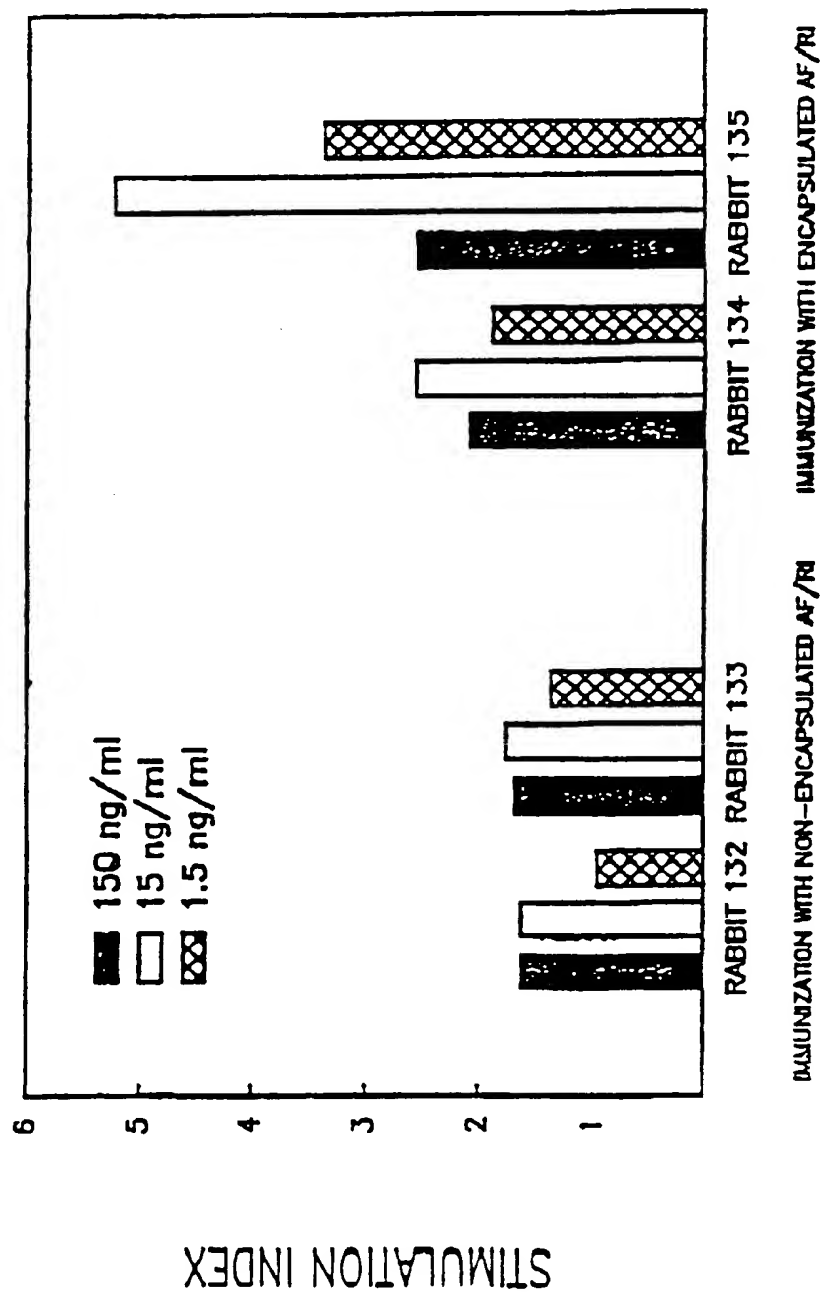
10 / 39

Fig. 80



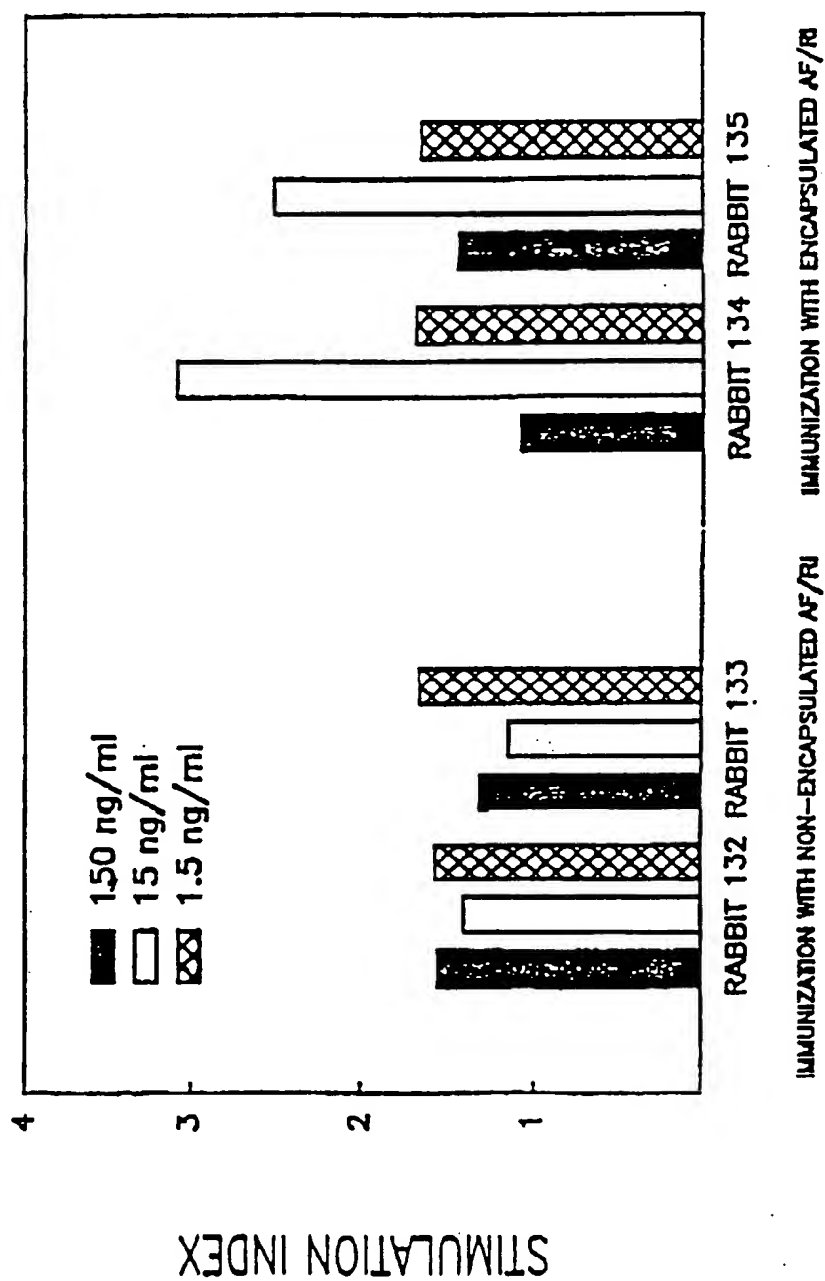
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Fig. 10



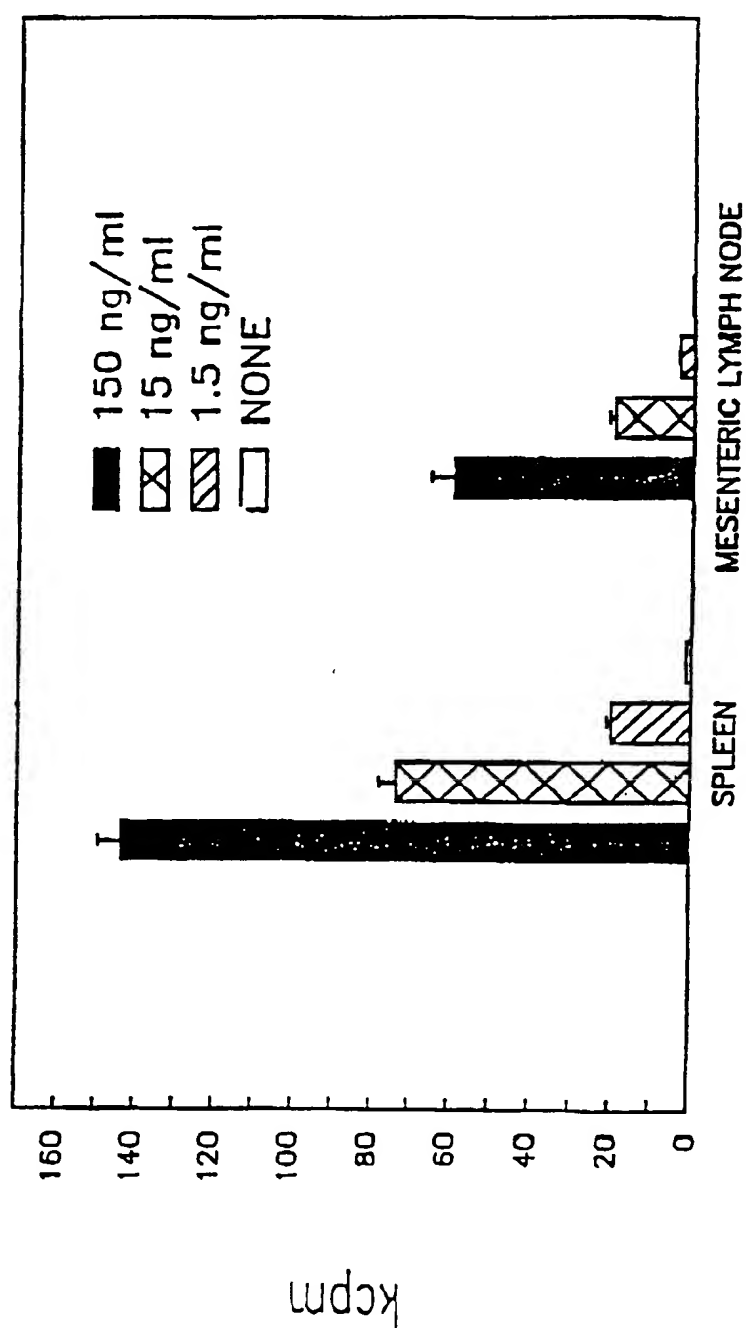
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Fig. 12



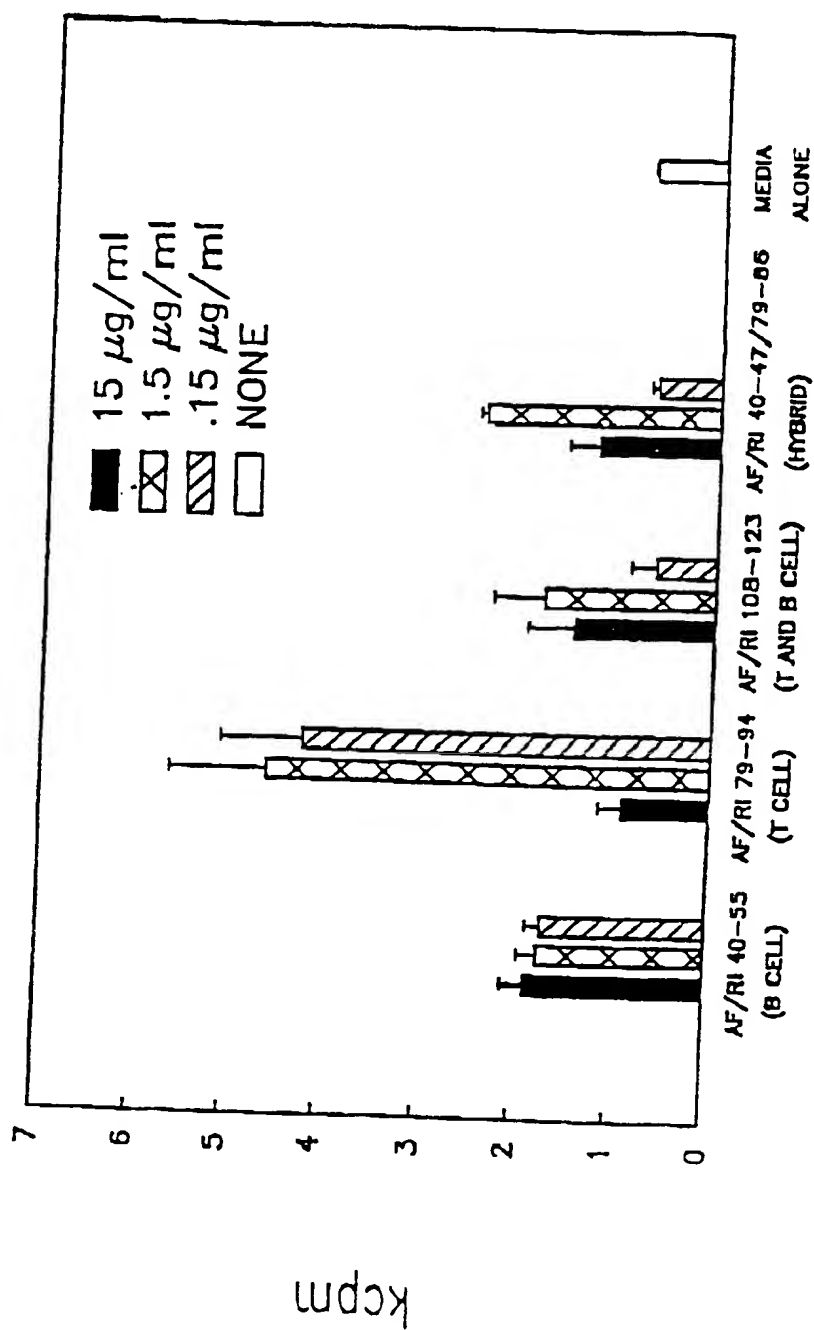
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Fig 13



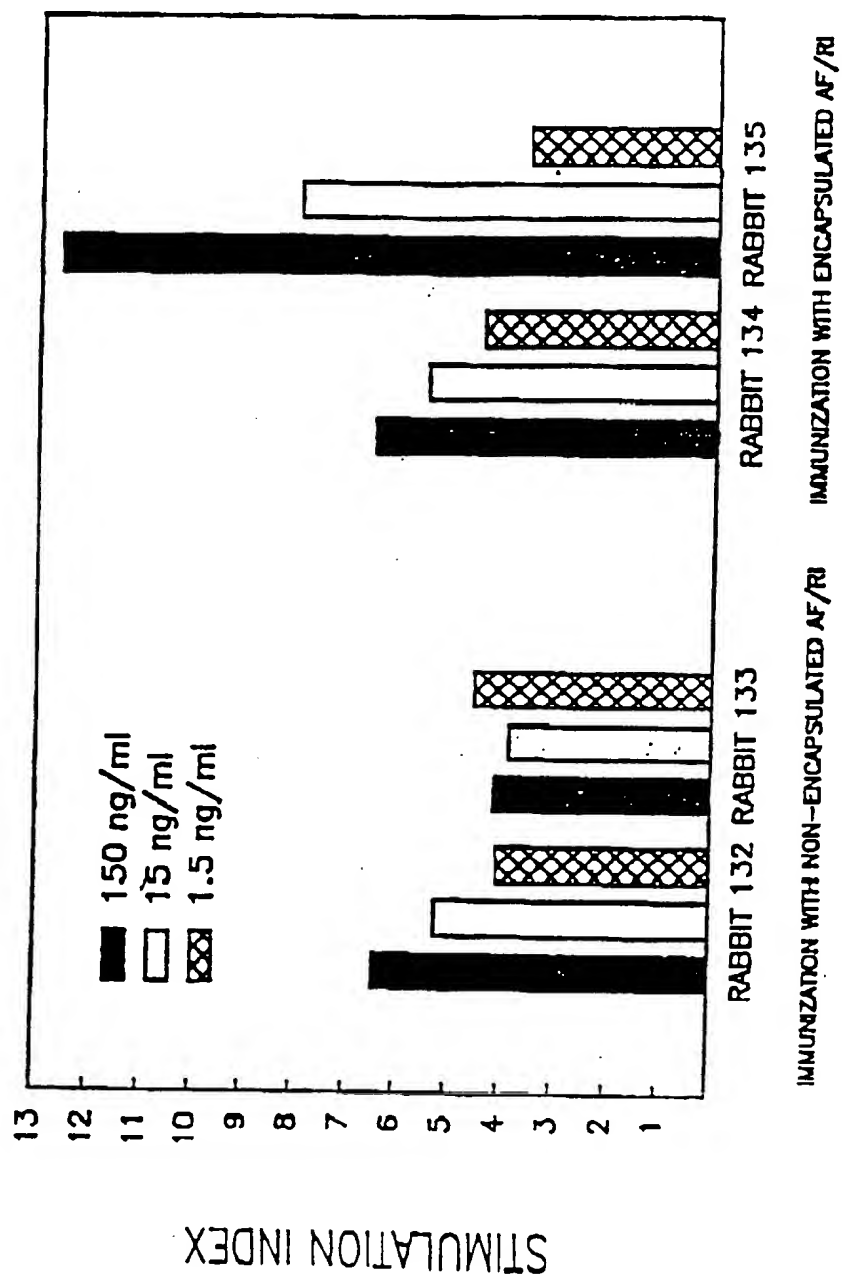
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Fig. 14

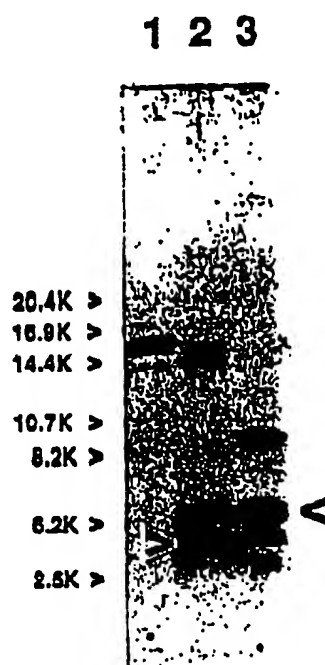


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Fig. 15



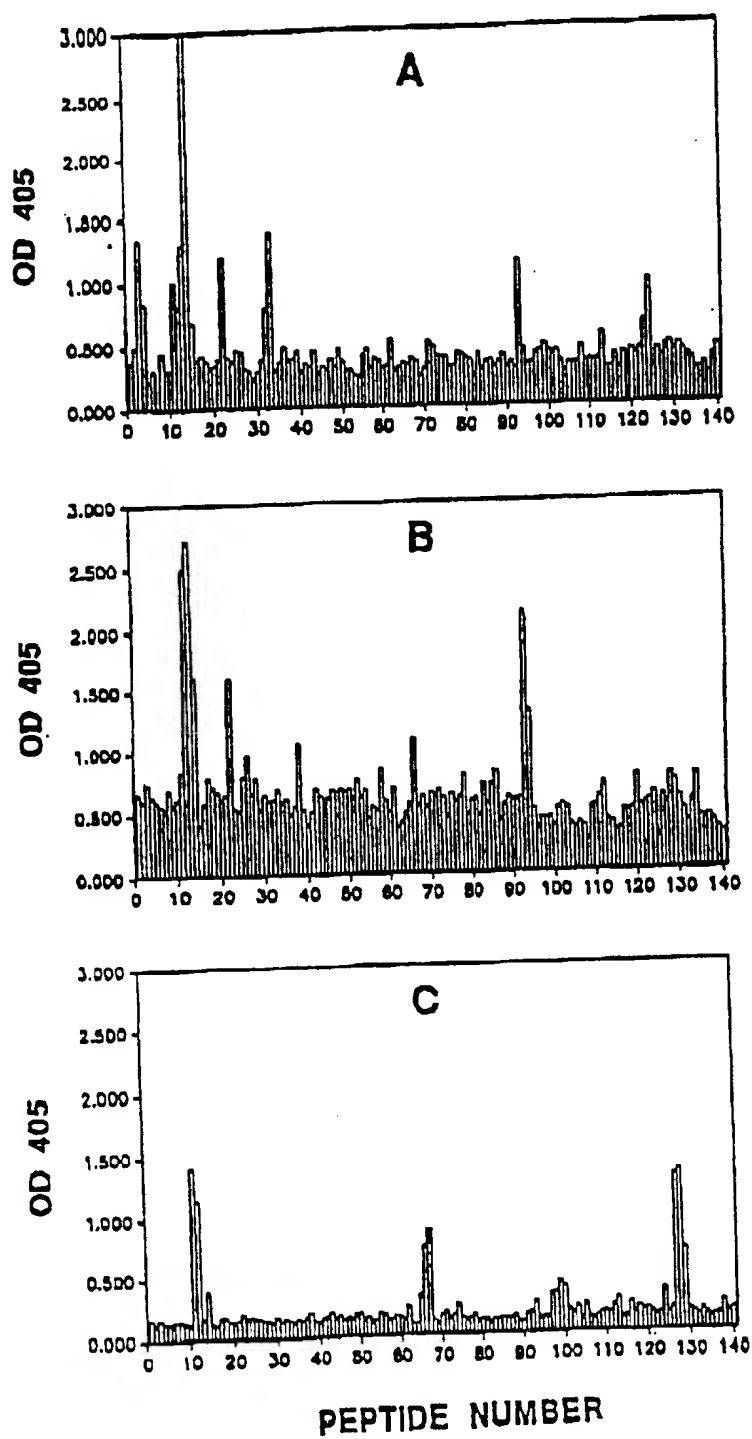
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A**B**

Lane 2	LADTPQLTDVLN <u>E</u> TVQMP	(52-79)
Lane 3	SYRVMTQVETN <u>D</u> ATKKVIV	(42-60)

Figure 16

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PEPTIDE NUMBER

Figure 17

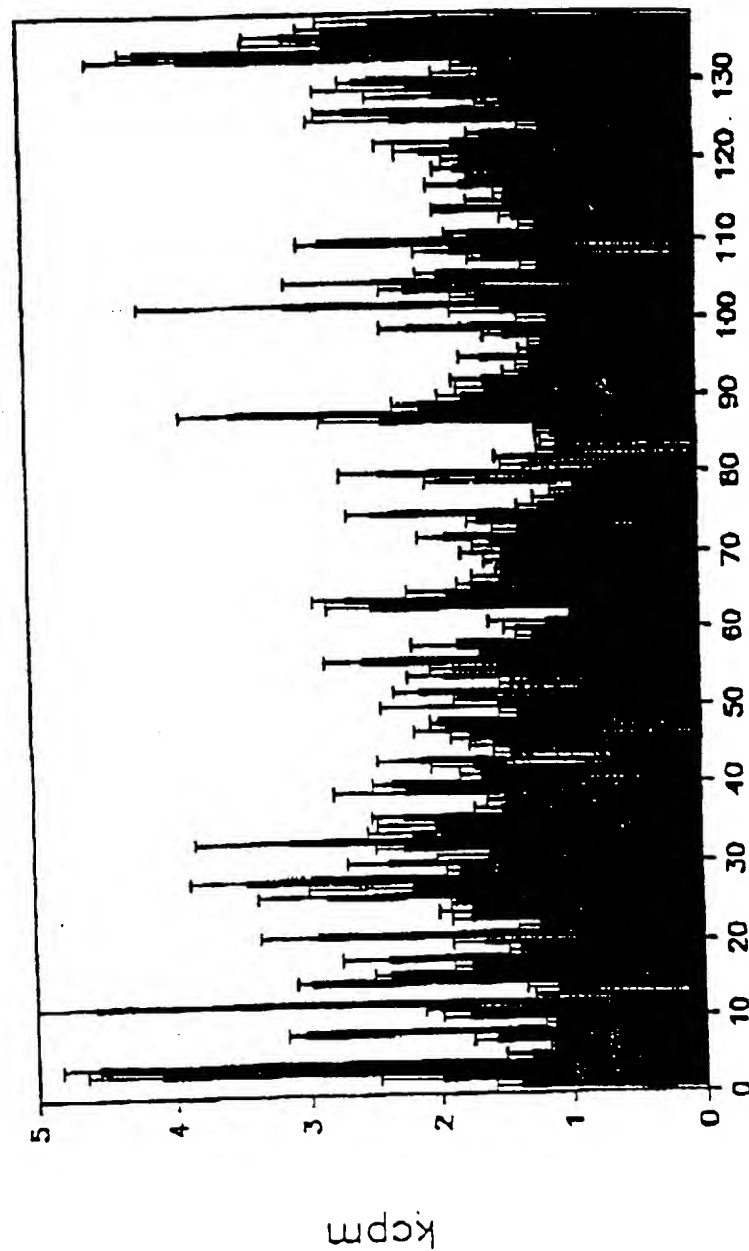
18 / 39

282	V E K N I T V T A S V	D P P V I D L L Q A D	G N A L P S A V	K L A Y S P A S K T F	Z E S Y R V M T Q V R	50
184D	V E K N I T V T A S V	D P P V I D L L Q A D	G N A L P S A V	K L A Y S P A S K T F	E S Y R V M T Q V R	
34	V E K N I T V T A S V	D P P V I D L L Q A D	G N A L P S A V	K L A Y S P A S K T F	E S Y R V M T Q V R	
282	T H D A T K K V I V K L A D T	P Q L T D V L N S T V Q M P I S	V S N G G Q V L S T T	A K E F E A A A		100
184D	T H D A T K K V I V K L A D T	P Q L T D V L N S T V Q M P I S	V S N G G Q V L S T T	A K E F E A A A		
34	T H D A T K K V I V K L A D T	P Q L T D V L N S T V Q M P I S	V S N G G Q V L S T T	A K E F E A A A		
282	L G Y S A S G V N G V S S S Q E L V I S A A P	K T A G T A P T	A G N Y S G V V S L V M T L G S			147
184D	L G Y S A S G V N G V S S S Q E L V I S A A P	K T A G T A P T	A G N Y S G V V S L V M T L G S			
34	L G Y S A S G V N G V S S S Q E L V I S A A P	K T A G T A P T	A G N Y S G V V S L V M T L G S			

Figure 18

Monkey 184 D (#11)

PROLIFERATIVE RESPONSES TO CFA/I SYNTHETIC PEPTIDE



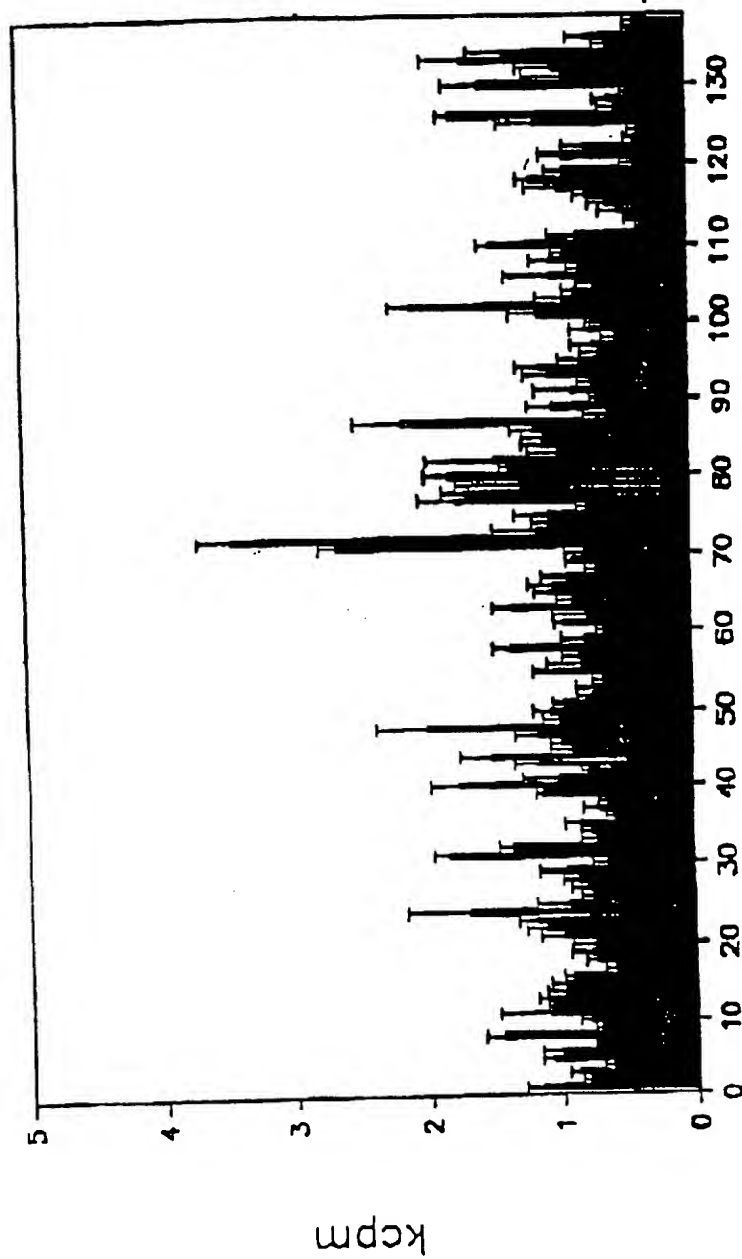
SYNTHETIC CFA/I DECAPEPTIDE, BEGINNING POSITION NUMBER FROM CFA/I SEQUENCE

Figure 19

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Monkey 34 (#2)

PROLIFERATIVE RESPONSES TO CFA/I SYNTHETIC PEPTIDE



SYNTHETIC CFA/I DECAPEPTIDE, BEGINNING POSITION NUMBER FROM CFA/I SEQUENCE

Figure 2a

Monkey 222(#3)

PROLIFERATIVE RESPONSES TO CFA/I SYNTHETIC PEPTIDE

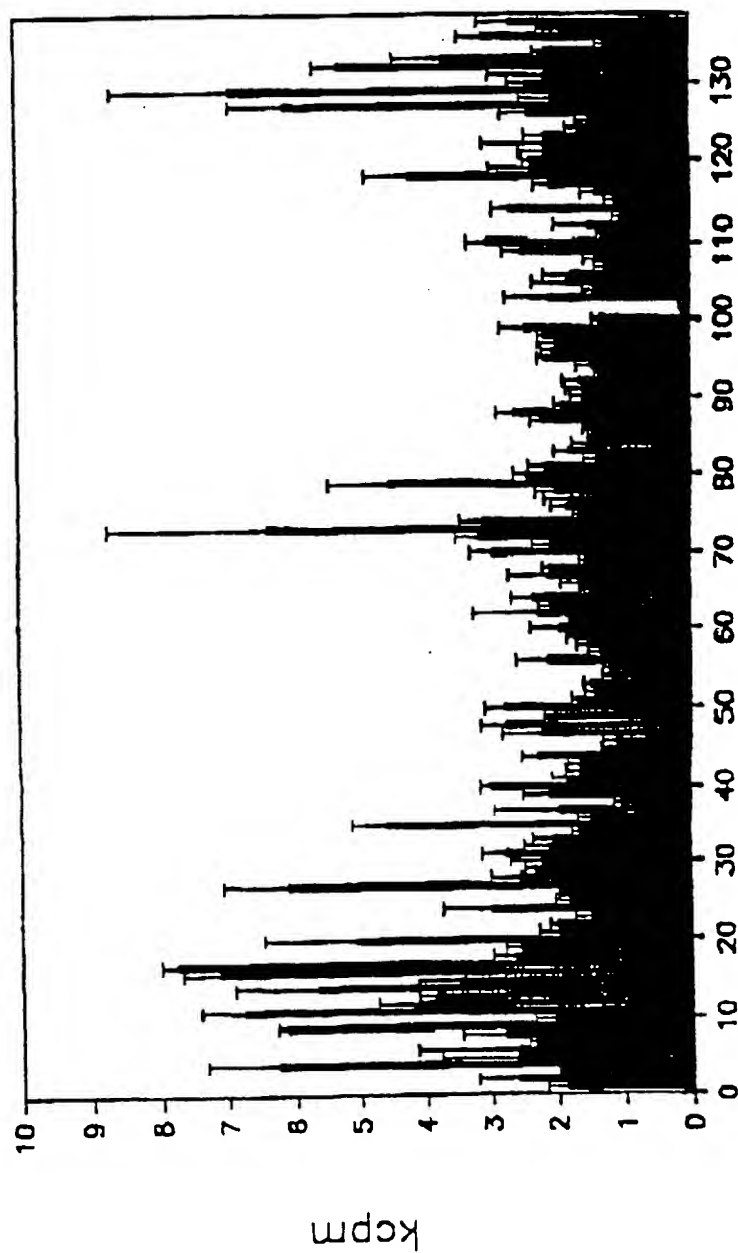


Figure 21

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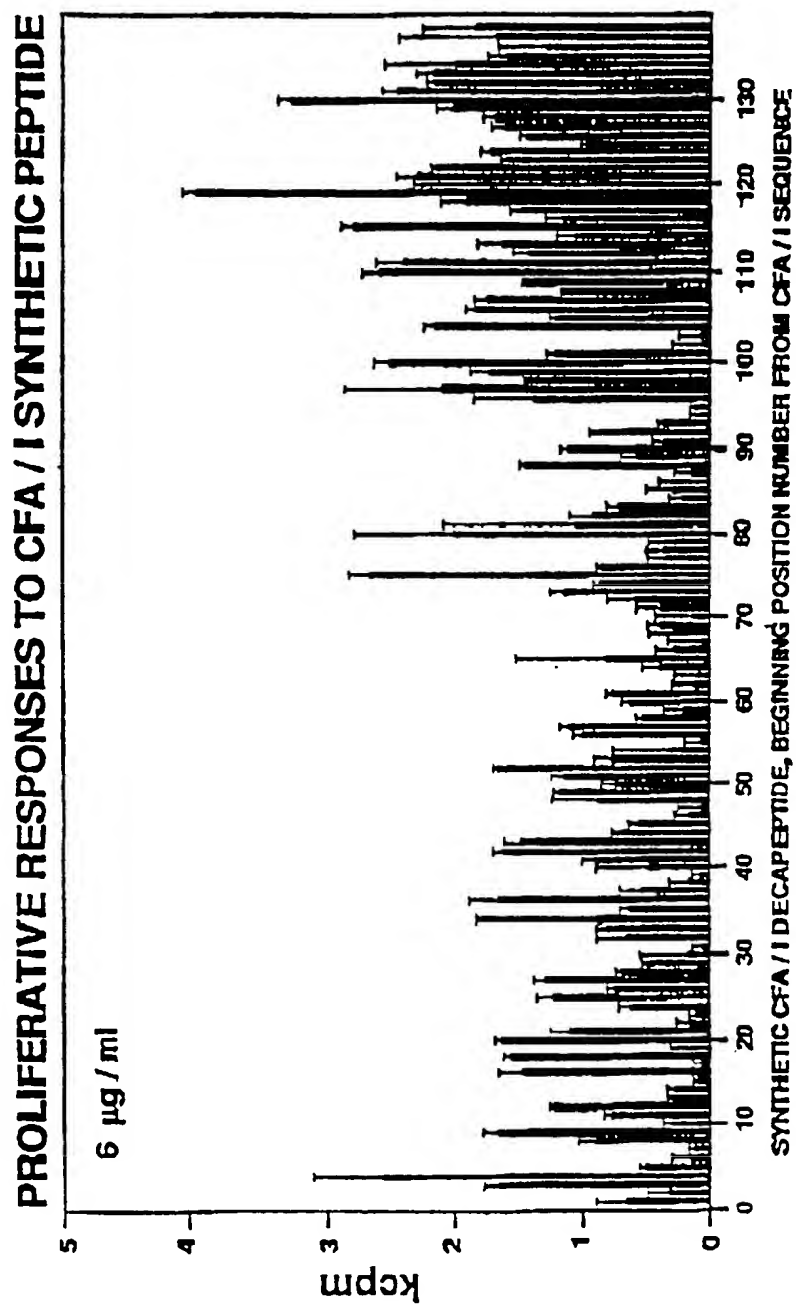


Figure 22

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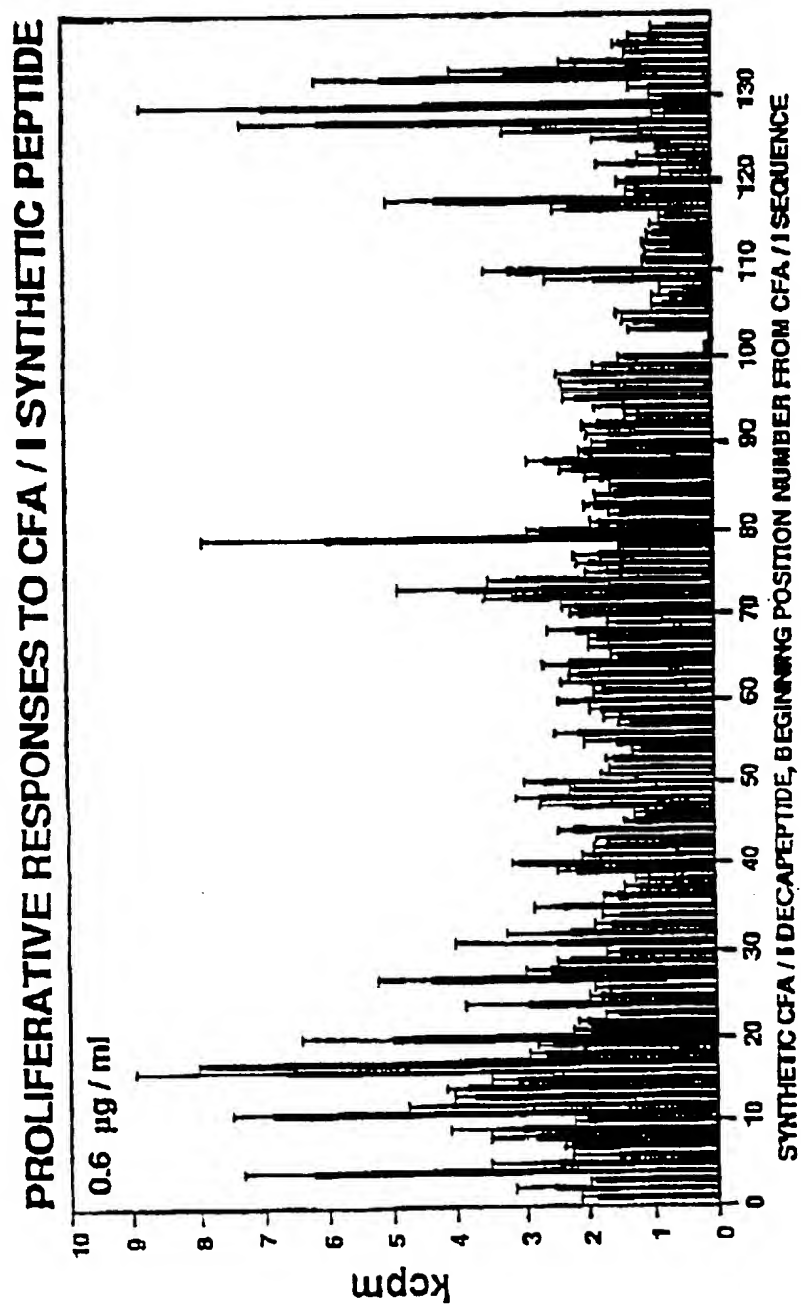


Figure 23

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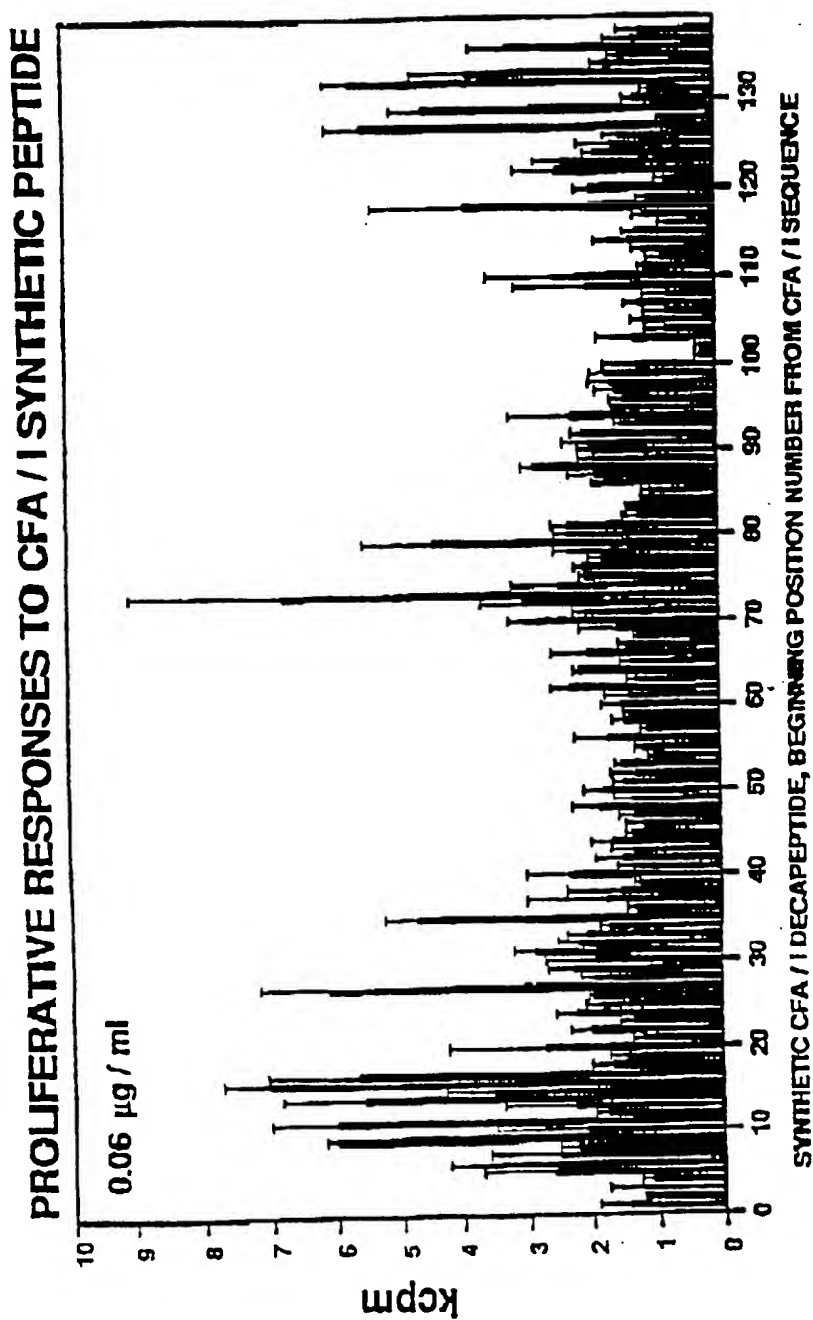


Figure 24

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kcpm

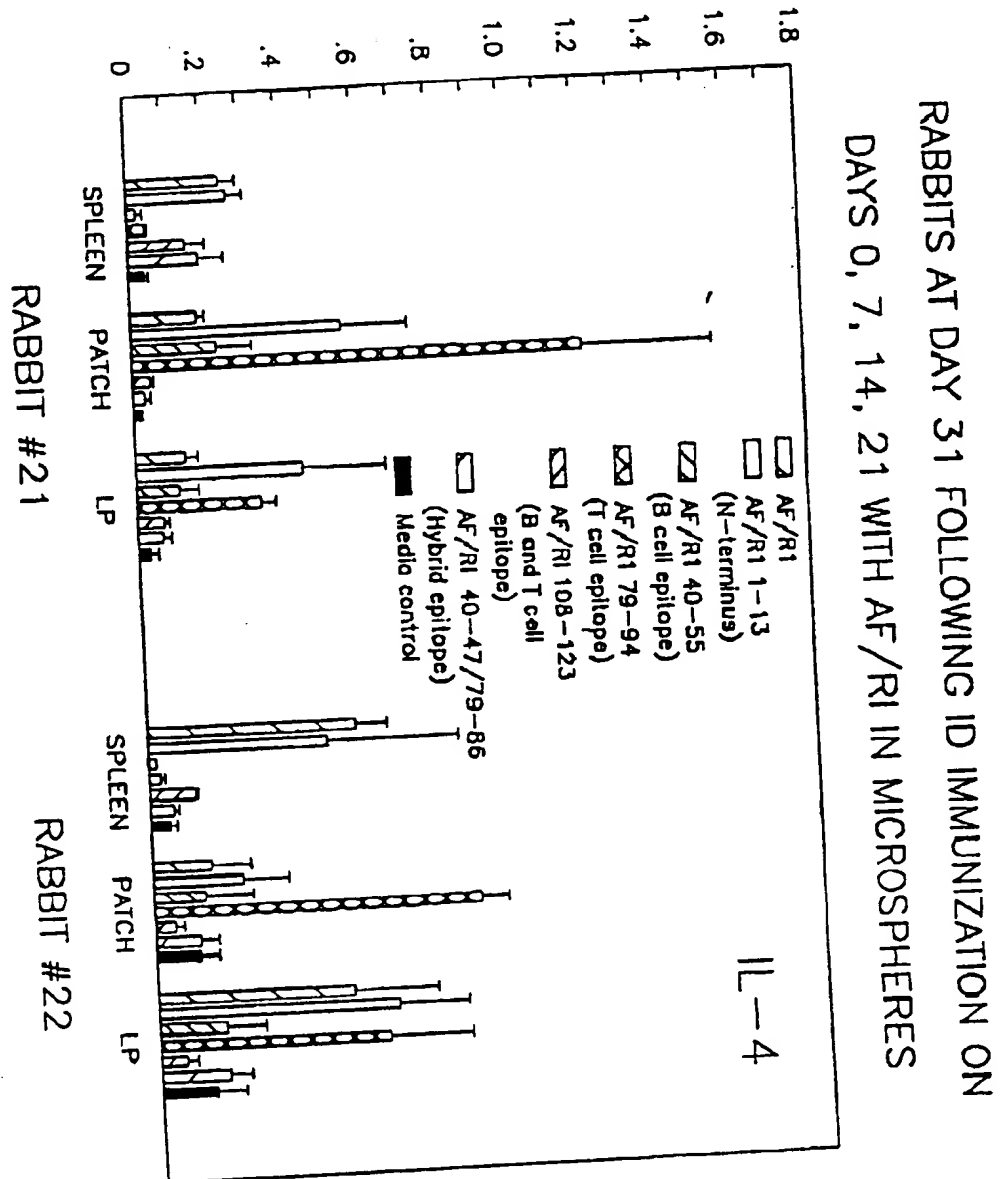


Figure 25

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RDEC-1 COLONIZATION

Immunized vs Unimmunized

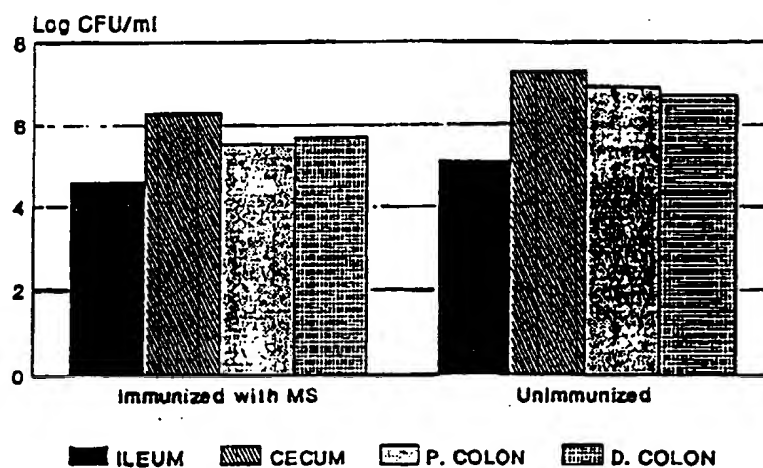
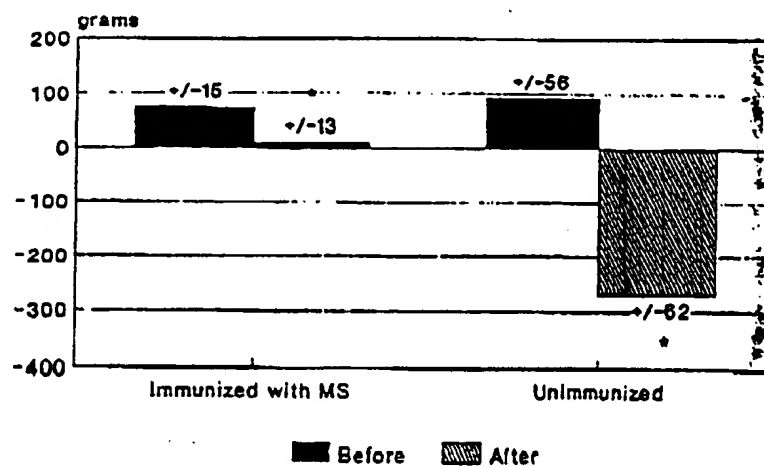


Figure 26

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WEIGHT CHANGES

Immunized vs Unimmunized

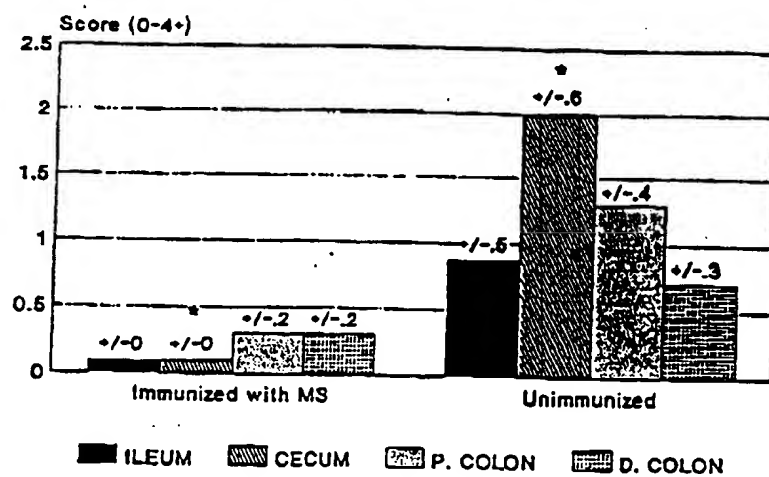


-P<.001

Figure 27

RDEC-1 ATTACHMENT

Immunized vs Unimmunized



* P<01

Figure 28

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Particle Size Distribution

CFA/II Microsphere Vaccine; Lot #L74F2

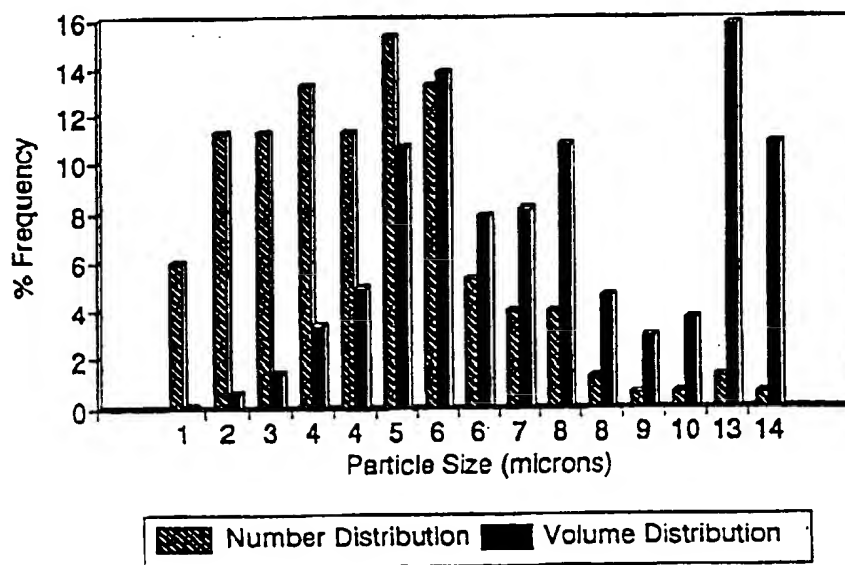


Figure 29

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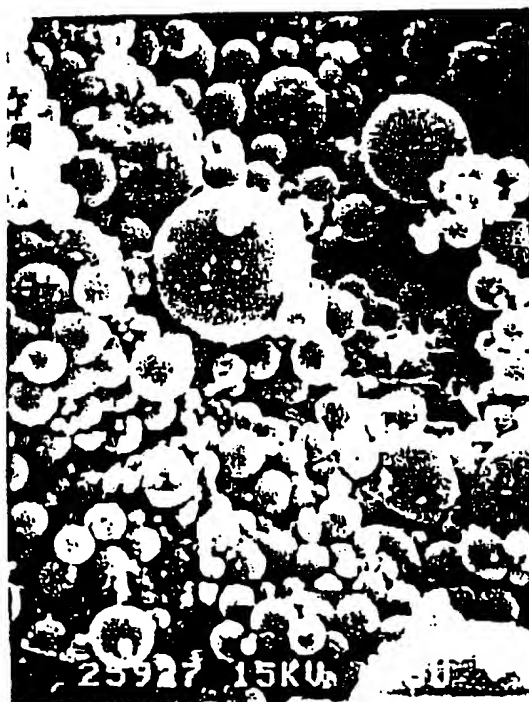


Figure 30

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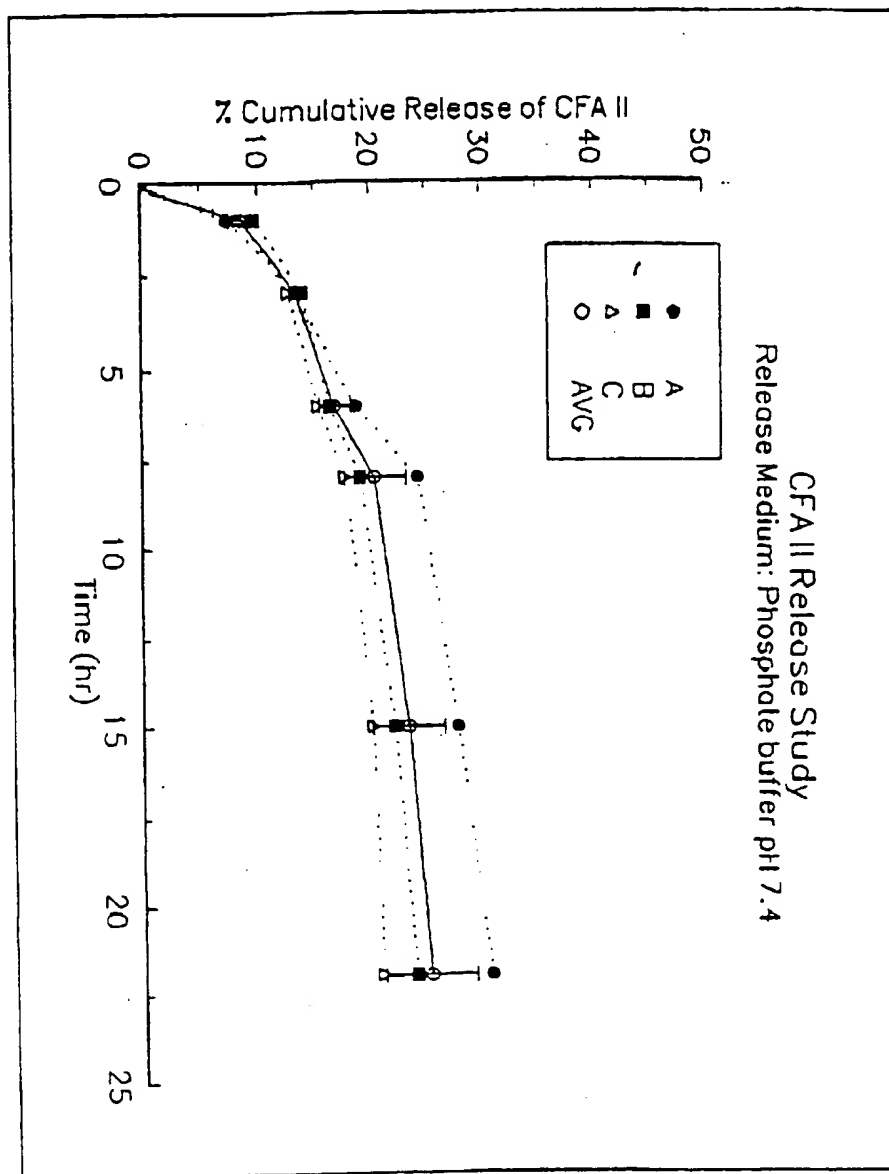


Figure 31

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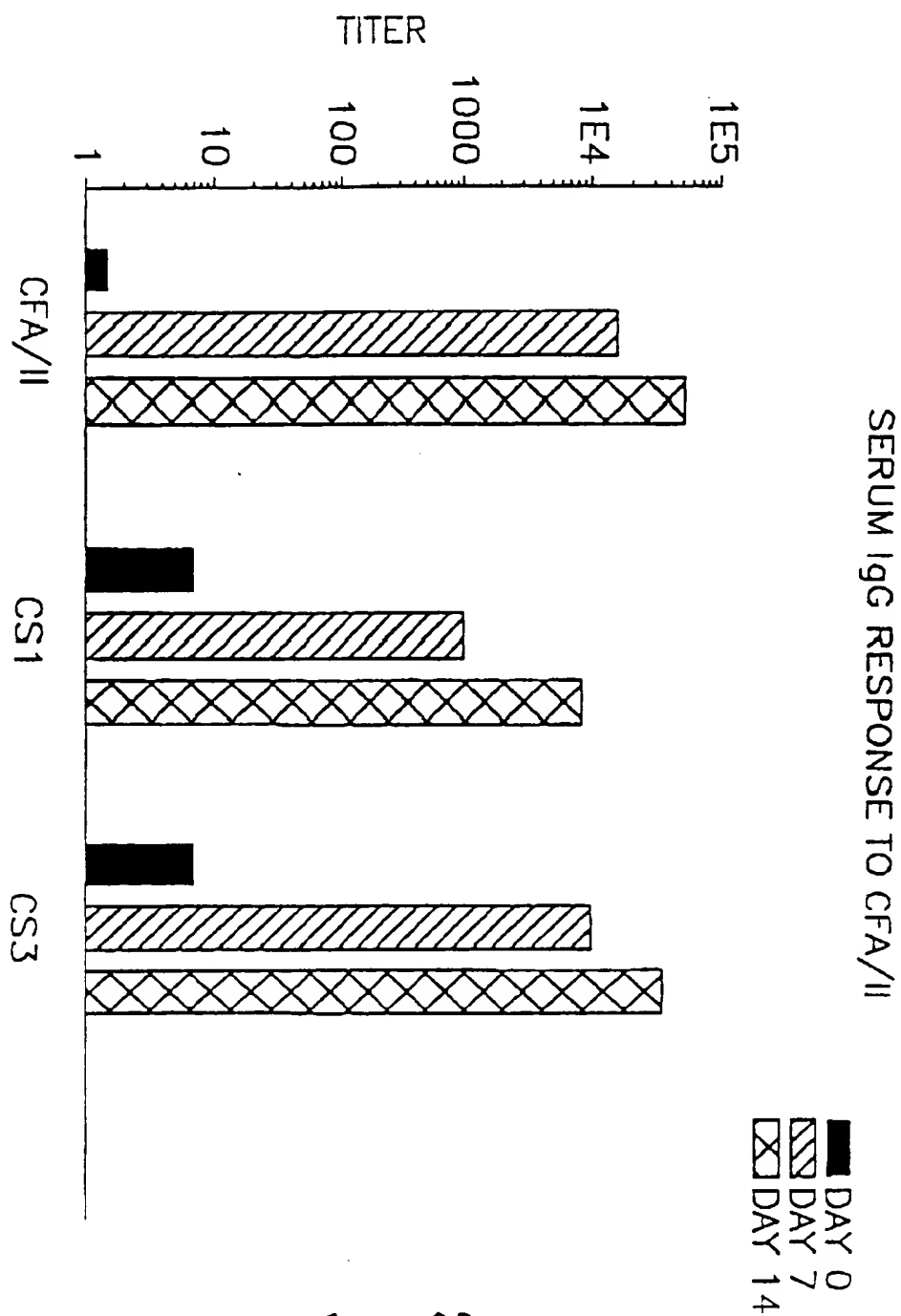
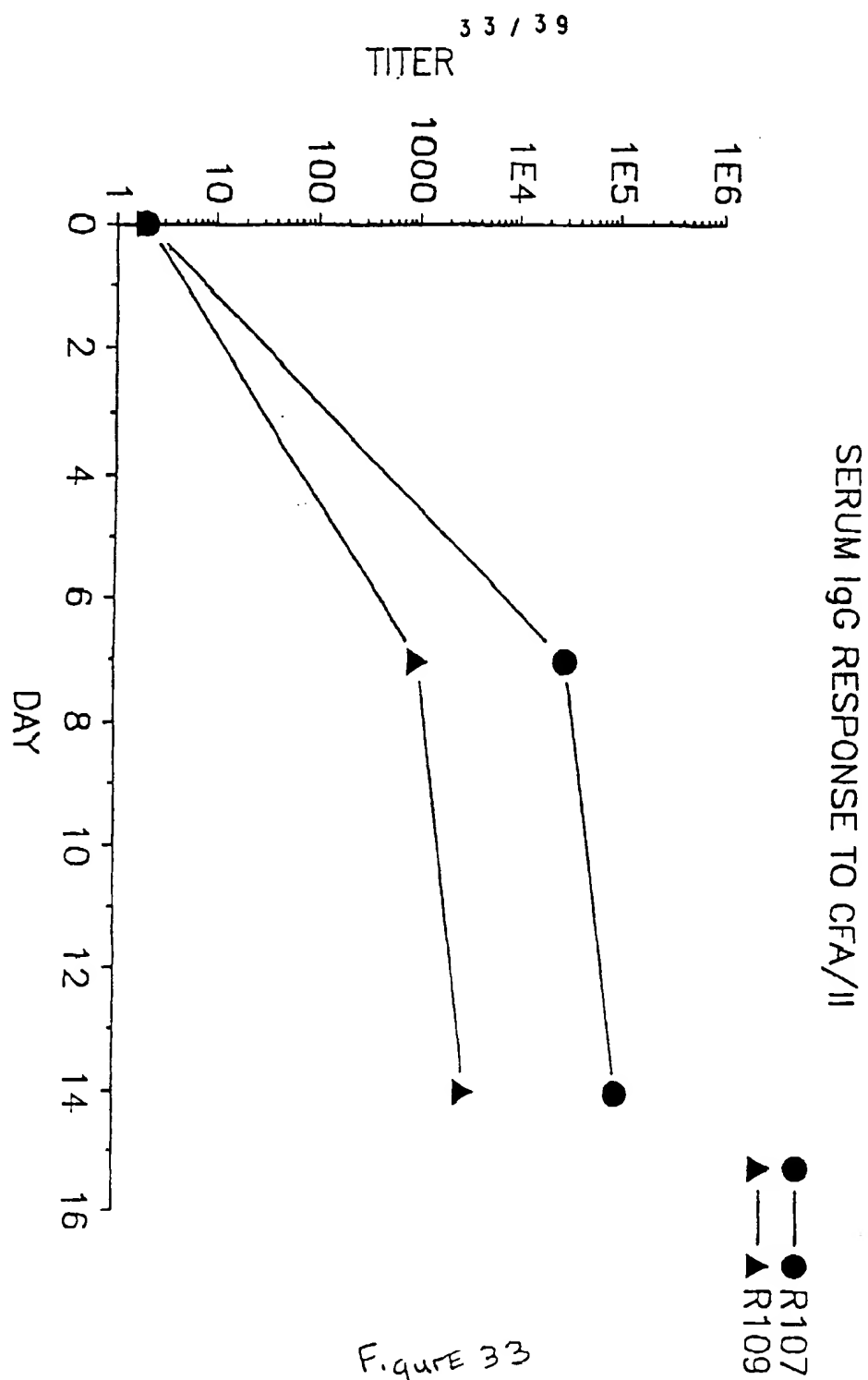


Figure 32



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LYMPHOCYTE PROLIFERATIVE RESPONSES

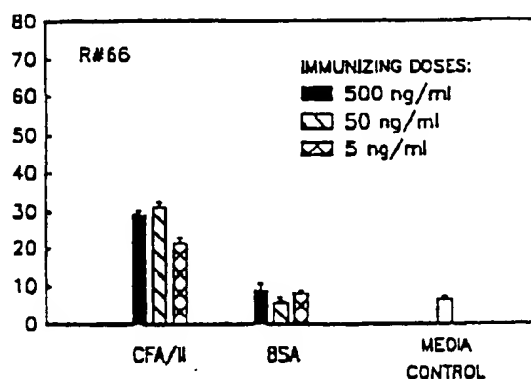


Figure 34 (b)

LYMPHOCYTE PROLIFERATIVE RESPONSES

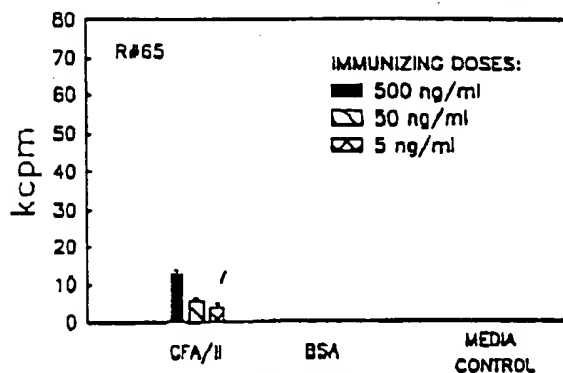


Figure 34 (a)

LYMPHOCYTE PROLIFERATIVE RESPONSES

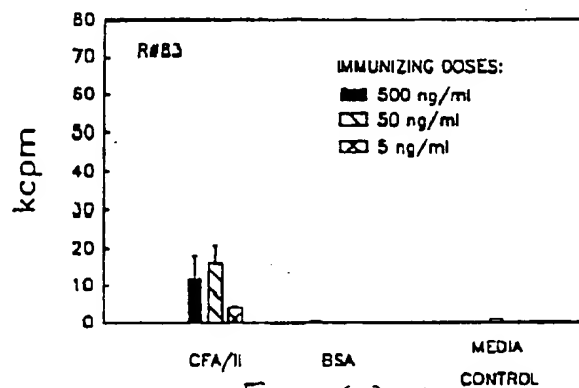


Figure 34 (c)

LYMPHOCYTE PROLIFERATIVE RESPONSES

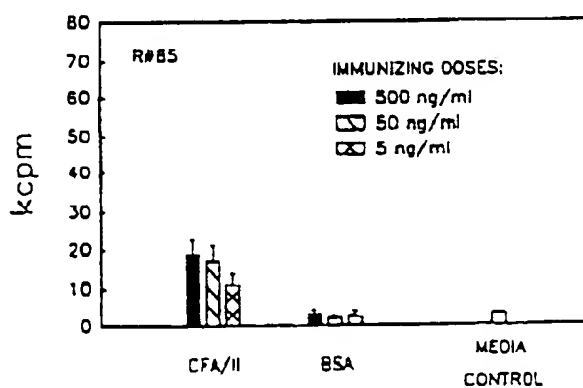
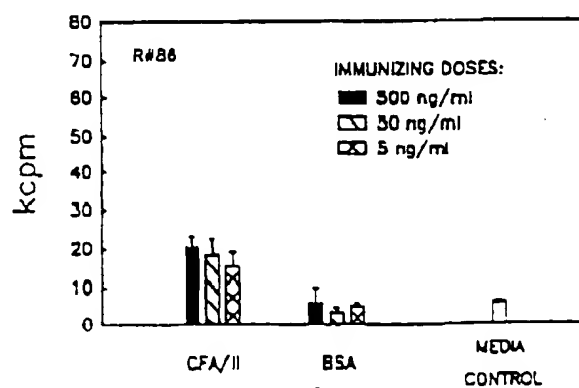


Figure 34 (d)

LYMPHOCYTE PROLIFERATIVE RESPONSES

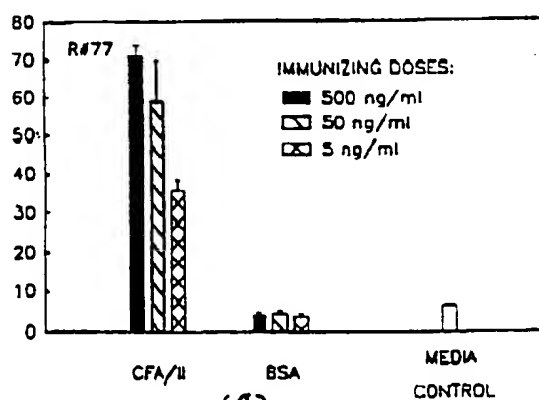


(e)

Figure 34

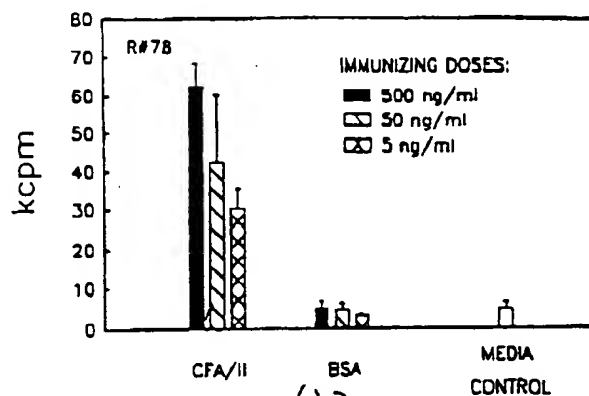
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LYMPHOCYTE PROLIFERATIVE RESPONSES



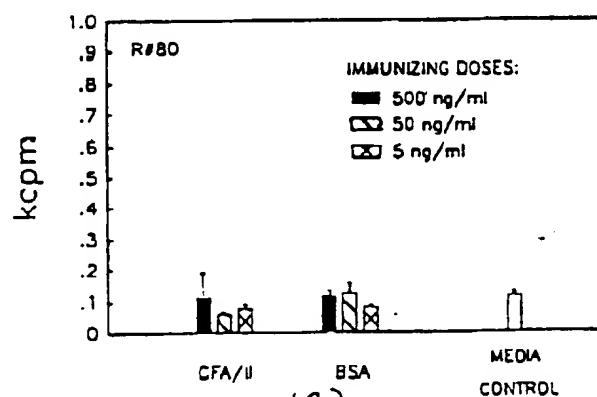
(a)

LYMPHOCYTE PROLIFERATIVE RESPONSES



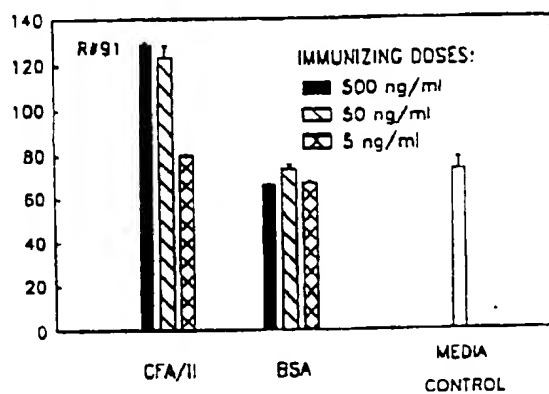
(b)

LYMPHOCYTE PROLIFERATIVE RESPONSES



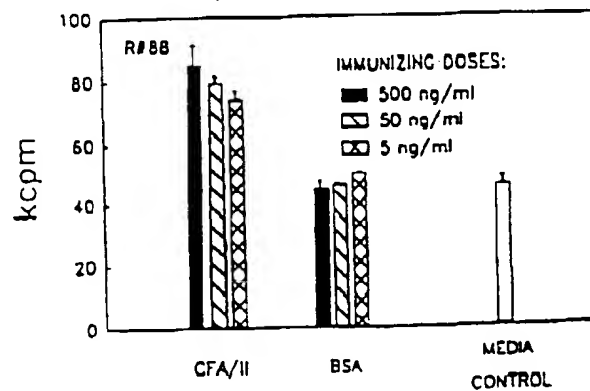
(c)

LYMPHOCYTE PROLIFERATIVE RESPONSES



(d)

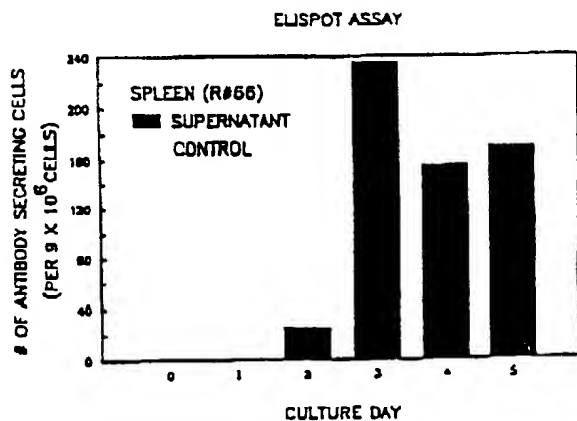
LYMPHOCYTE PROLIFERATIVE RESPONSES



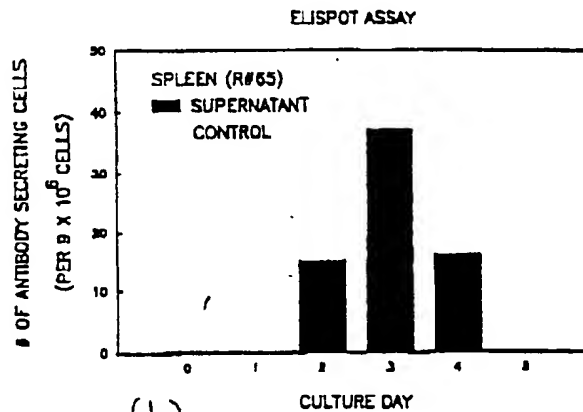
(e)

Figure 35

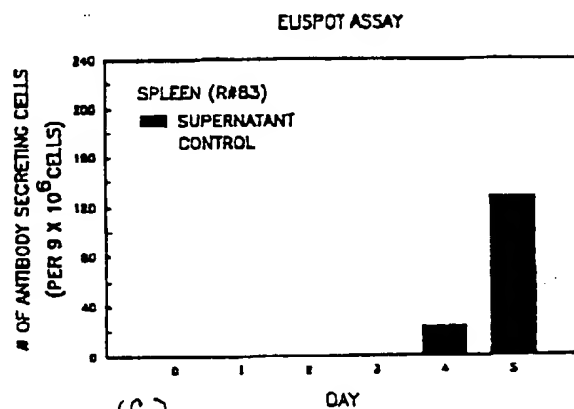
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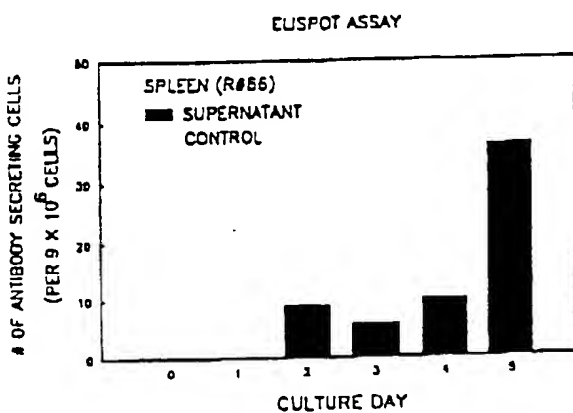
(a)



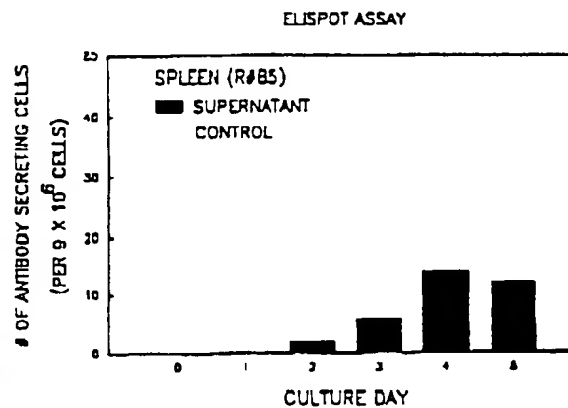
(b)



(c)



(d)



(e)

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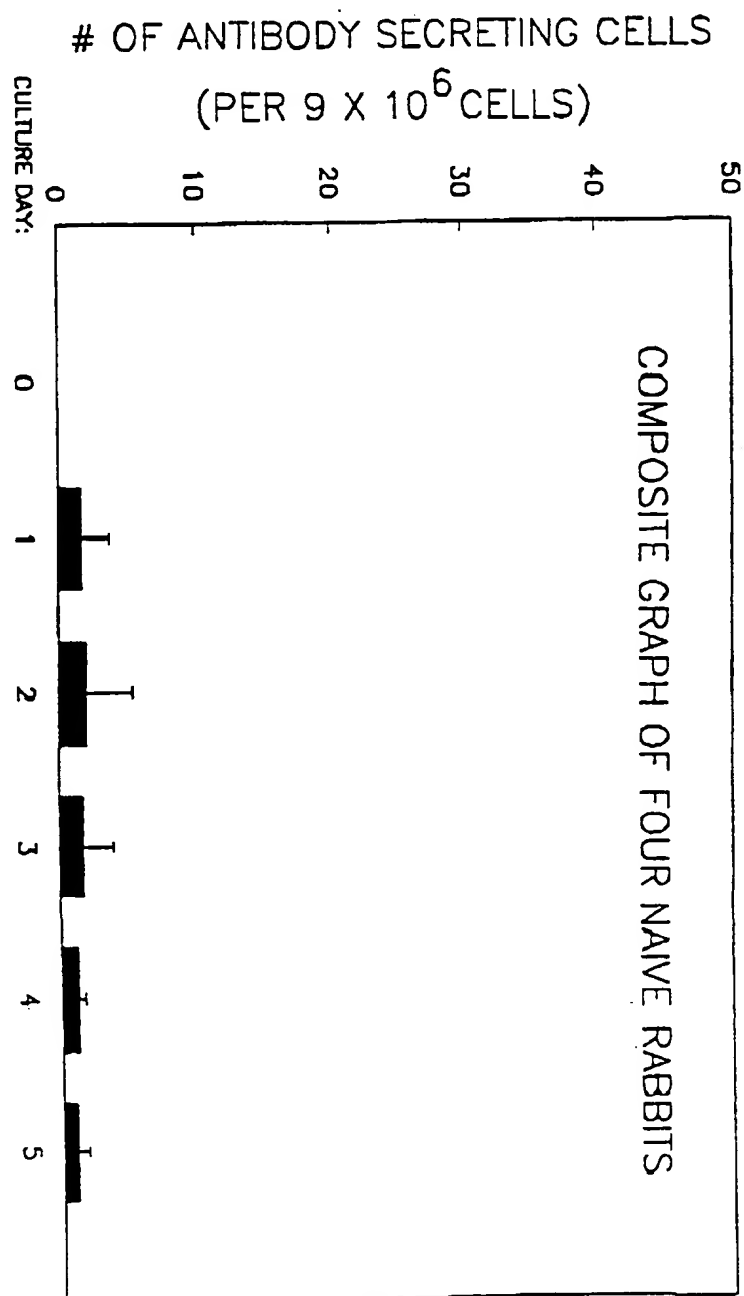


Figure 37

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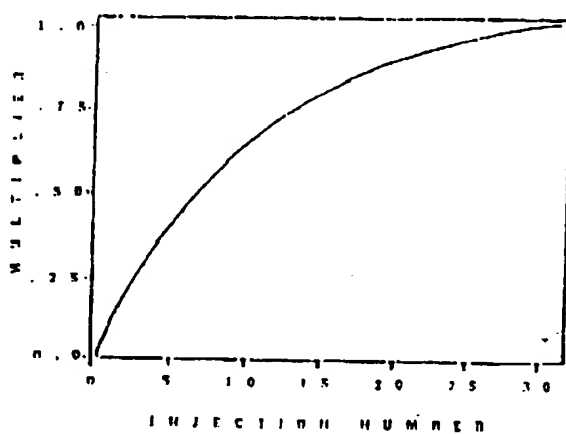


Figure 38

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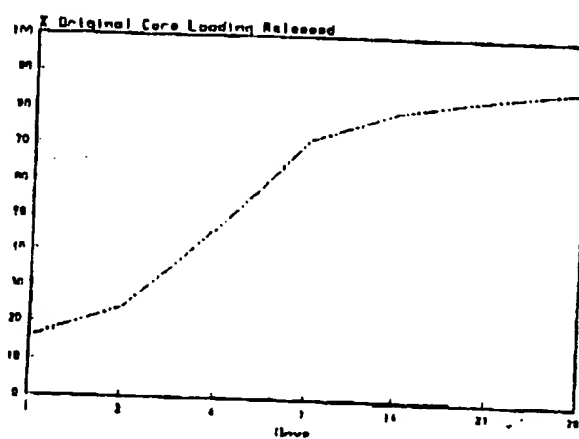


Figure 39

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/02536

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 39/02, 9/26

US CL : 424/85, 88, 89, 92, 417, 422, 450, 458, 469

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85, 88, 89, 92, 417, 422, 450, 458, 469

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

GLUCOID (P) LACTIDE (P) ORAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,897,268 (TICE ET AL.) 30 JANUARY 1990, COLUMN 2, LINE 6 TO COLUMN 3, LINE 25.	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

16 JUNE 1994

Date of mailing of the international search report

JUN 23 1994

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Washington, D.C. 20231

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Authorized officer

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